MITOCHONDRIAL ANCIENT DNA ANALYSIS OF LAWSON CAVE BLACK BEARS (URSUS AMERICANUS)

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Master of Arts

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

MITOCHONDRIAL ANCIENT DNA FROM LAWSON CAVE BLACK BEARS (URSUS AMERICANUS)

presented by Corey M. Hudson,

a candidate for the degree of Master of Arts, and hereby certify that, in their opinion, it is worthy of acceptance.

Professor R. Lee Lyman

Professor Lori S. Eggert

Professor Gregory E. Blomquist

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First and foremost, I feel I'd be remiss if I didn't acknowledge the tremendous contribution Dr. Lori Eggert made to the completion of this thesis. I started this project with very little hands on experience in a molecular lab and none working with ancient DNA. Lori has been an amazing mentor, guiding me through both basic and more advanced techniques, and allowing me enough leeway to learn a great deal (many times through trial and error). Lori also made available funds from the University of Biological Sciences Lab Initiation Fund, without which this project would not have been possible.

In the three years since I arrived at the University of Missouri, Dr. Lee Lyman has been a first-rate mentor and advisor. Lyman's prolific publication of papers has inspired me to write, and his dutiful work editing both my writing and my line of logic is one of the only things that made writing this thesis possible. The idea for this project came out of a short series of conversations between Lyman and me. I knew that I wanted to study the intersection of conservation and paleozoology, it was Lyman however who suggested that I use my moderate experience in biology to address the topic through ancient DNA. Lyman showed an enthusiasm for this project and trust in my abilities that I have come to appreciate as a rare combination in advisors.

Many of the modern data points and indeed the original project, studying the modern black bear population in Missouri, came out of work done by an undergraduate in Lori's lab: Kaitlyn Faries. Kaitlyn's work generating genetic data gave this thesis its conceptual foundation. Kaitlyn was also tireless, answering my many questions, fielding concerns, providing me samples, sharing data, and teaching me a number of lab techniques.

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ABSTRACT

The distribution of black bear (*Ursus americanus*) in southern and central Missouri has been controversial. This controversy centers on two questions: 1) Where does the historical species fit into the continental phylogeography; 2) Are the contemporary black bears native to the region, or the result of an in-migration of black bears translocated into Arkansas? To answer these questions I extracted DNA from 10 black bears, collected from Lawson Cave, an Historical Era (0-550 year old) site in central Missouri. These bears are the most recent samples that can be unambiguously identified as native to Missouri. I successfully amplified the control region of the mitochondria of four of the 10 samples. Two of the four samples are exact matches to a known haplotype, extending from Minnesota to Mexico. Using modern samples and sequences from central North America I created a phylogeny that grouped into two clades. All of the samples from Lawson Cave grouped into clade 1. This suggests that this clade is recently native to Missouri. I also compared samples collected from a study of modern Missouri black bears. These bears fell into both clade 1 and clade 2. This study was unable to determine whether certain modern bears belong to clade 1 as a result of *in situ* mitochondrial continuity, or because of the widespread distribution of this clade throughout central North America. These results suggest that although certain bears belong to a clade native to Missouri, many also belong to a group not known from Missouri's historic past.

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-CHAPTER ONE-

INTRODUCTION

The specific role of humans in determining modern distributions of animal taxa across the landscape is one of the most hotly debated issues in conservation biology. These debates center on two primary themes: 1) In what ways have the effects of anthropogenic agency been complicit in the systematic decline of biodiversity on a regional and global scale (Gaston 2006; Hughes et al. 1997), and 2) How has the introduction of non-native/alien species by humans impacted the 'natural' (and presumably sustainable) functioning of ecosystems (Mack et al. 2000)? Attempts to answer these two questions have led conservation biologists to incorporate the seemingly disparate fields of molecular ecology and paleozoology (Frankham et al. 2002; Lyman and Cannon 2004).

In essence, conservation biology is focused on sustaining ecological diversity and the historical/evolutionary processes that maintain it (Moritz 2002; Soulé 1985). An understanding of the genetic basis for species' inter- and intra-specific interactions as well as their interaction with the environment is therefore essential in order to infer the distribution and causes of diversity, as well as to provide its fundamental metrics (Reed and Frankham 2003). Paleozoology is also important in conservation biology because it is *empirical natural history.* Unlike other fields, which also study natural history (e.g., phylogenetics, systematics, evolutionary ecology, and biogeography), only paleozoology studies animal history in the direct observational sense of examining bones, teeth, and shells in their geological/spatiotemporal contexts. In other words, in terms of promoting the goals of conservation biology molecular ecology provides the measures and causes,

while paleozoology provides the tests of hypotheses regarding species occurrence and diversity in a spatiotemporal context, particularly over longer periods of time than provided by the historical record or reasonable through traditional observational studies in ecology.

The fields of molecular ecology and paleozoology have been recently integrated through the study of ancient DNA (aDNA). Ancient DNA refers to a set of methods that are used to extract and amplify DNA from materials that have had the quality and quantity of their genetic material compromised through the passage of time (Hofreiter et al. 2001). Studies run the gamut from the analysis of a short sequence of mitochondrial DNA (225 base pairs) from 50-70 year old museum specimen kangaroo rats (*Dipodomys panamintinus*) (Thomas et al. 1990) to the characterization of the complete nuclear genome (4.168 gigabase pairs) of a ~20,000 year old (18,454±70 ^{14}C yr B.P.) woolly mammoth (*Mammuthus primigenius*) extracted from permafrost (Miller et al. 2008). The unifying theme in these studies is that they are both molecular and express a depth of time. It is now possible, using these methods, to incorporate measures from molecular ecology with materials from paleozoology. The number of studies that have incorporated paleozoology and molecular ecology is growing and this thesis represents a further attempt at connecting these fields with conservation questions.

Purpose of the Study

Historical records from county records and the oral accounts of fur trappers indicate that black bears (*Ursus americanus*) were found abundantly in Missouri throughout the 18th and 19th centuries (McKinley 1962). Additionally, Historic Era (sites

created between 0 and 550 years before the present) archaeological sites containing skeletal remains of black bears occur throughout the central Midwest (Figure 1, FAUNMAP Working Group 1994). However, as the Missouri hardwood forest shrank to 2% of its original area (Korte and Frederickson 1977), the black bear was believed to have been completely extirpated in Missouri by 1931 (Bennitt and Nagel 1937). This belief has been a point of contention, with some arguing that a small residual population remained in Missouri's Ozark region (MDC 1993).

What is currently known is that late in the twentieth century there is a population of black bears in southern Missouri (Smith and Clark 1994). The origin of these bears is problematic because between 1959 and 1967 the Arkansas Game and Fish Commission translocated 254 black bears from Minnesota and Manitoba, Canada, into the Ozark and Ouachita Mountains in western Arkansas (Smith and Clark 1994). Because of this translocation, the source of the modern population of Missouri black bears is unknown. Knowing whether the modern population is native or translocated could help conservation professionals evaluate black bear management priorities (Clark et al. 2002). Ultimately, understanding the historical origin of the modern bears will help conservation professionals make more informed decisions and evaluate the decisions they have already made with regard to what is believed to be a growing population of black bears in Missouri (MDC 2008).

This analysis was initiated to provide a genetic snapshot of Historical Era black bears in Missouri. Analyzing the chosen collection has involved two interrelated components: (1) development of a chronology of ursid remains and (2) characterization of a portion of mitochondrial DNA, collected from central and southern Missouri black

bears represented by remains from an Historical Era cave site as well as a sample from the modern population. The purpose of the research reported in this thesis is to characterize the genetics of a specific collection of Missouri black bears, with two goals in mind: (1) To determine where Missouri black bears fit into the biogeographic distribution of North American black bear haplotypes, and (2) To determine the genetic impact of the 1960s black bear reintroduction on the current population of Missouri bears.

For this study I extracted and analyzed genetic material from a paleozoological collection of black bear specimens excavated from a natural-trap cave site – Lawson Cave – located in central Missouri (Figure 2). Natural-trap cave sites accumulate their faunal materials as animals unknowningly become trapped in them, often falling to their death. In paleozoology, the means by which faunal materials accumulate at a site are known as its taphonomy (Lyman 1994b). The nature of this particular site's taphonomy guarantees that the black bears from this site lived and died in central Missouri (Wolverton 2006). This contention is supported by the depth of the cave, the narrowness of its entrance, the well-reported attraction of black bear to the smell of carrion, and the lack of human-modified artifacts – which might otherwise suggest that the black bears had been disarticulated and carried to the site.

The initial radiocarbon dates for the Lawson Cave bears range from 233±39 to 170±60 (^{14}C yr B.P.) (Wolverton 2001). This range of dates places these specimens in Missouri's historical period (post AD 1541) (O'Brien and Wood 1998). These specimens are significant because, given their age, they represent the most recent population of black bears that can be unambiguously identified as 'native' to Missouri. These bears,

therefore, serve as an important proxy for the population of black bears that existed immediately prior to the decimation of Missouri's hardwood forests.

Analysis of the Lawson Cave bears will therefore provide a point of genetic comparison with the extant Missouri population. It will also serve the broader goal of contributing to our knowledge of the population structure of the North American black bear. Several studies have examined the control region of black bear mtDNA, but none of them have examined black bears in Missouri (Byun et al. 1997; Cronin et al. 1991; Onorato et al. 2004; Paetkau and Strobeck 1996; Robinson et al. 2007; Stone and Cook 2000; Wooding and Ward 1997). This area is informative because it lies between two North American mtDNA lineages within Wooding and Ward's clade A (1997). Wooding and Ward (1997) found two distinct clades (clade A and clade B), one in eastern, central and western North America and one only in northwestern North America. The timing of the split between these two clades (most recent common ancestor 1.8 ± .8 mya) led Wooding and Ward (1997) to propose that the split is the result of forest refugia during the Wisconsin ice age. Within the first of the clades (Clade A) there is a more recent phylogeographic grouping between eastern and western lineages. The margins of these lineages are unclear, in large part because of the lack of studies in central North America. This study, therefore seeks to understand where native Missouri black bears fit into these lineages and to help paint a more complete picture of black bear phylogeography. The difficulty that biogeographers have had fitting Missouri black bears into a general biogeographic scheme is apparent when comparing North American black bear distribution maps, and in particular the recorded presence/absence of black bears in Missouri. Figures 3-6 show exemplary distribution maps that were made between 1981

and 2000. These maps show that the known distribution of black bears is unclear, and for Missouri, these four maps show three different distributions. Fitting Missouri into this phylogeographic picture requires aDNA studies because no extant Missouri black bears can as of yet be unambiguously identified as native to the region.

Collection Description

The Lawson Cave site and its constituent faunal materials have been previously described by Wolverton and Lyman (1998, see also Wolverton 1996). The site occurs in the Three Creeks Conservation area approximately 4 km south of Columbia, MO, city limits (lat: 38º50'36.50" N, long: 92º17'05.98" W; Township 47N, Range 12W, Section 21, NW ¼ Section, SW 1/16 Section; Figures 2 and 7). The site was originally excavated between 1947 and 1959. Survey work was initiated in 1947, followed by an intensive four day survey in 1954, and the removal of 10" of surface soil substrate in 1958 and the removal of 28" in 1959. Sedimentary materials were screened (1/4" screen mesh) after being bucketed out of the cave, but no effort was made to record either the vertical or horizontal provenience of the faunal specimens (Figure 8). Recording locational provenience may have only been of limited usefulness, however, since the frequency of faunal material (suggested from the site excavators' notes) and the depth of sediment both peaked directly under the cave's entrance and tapered off toward the margins (Figure 9). This distributional pattern suggests that the way in which we typically present and analyze stratigraphy, as a layered sequence, with more recently deposited materials occurring on progressively higher layers and with each layer representing a roughly contemporaneous association of material, may be confounded by faunal materials being

aggregated at a specific point, then rolling or sliding down the margins. In this case vertical provenience (an archaeologist's primary basis for evaluating changes over time) and horizontal provenience (an archaeologist's primary tool for measuring temporal association and for evaluating the interrelatedness of two objects, in this case the ability to presume that the nearness of two specimens means that they were from the same individual) are probably of little use.

The site contains no less than 10 black bears (number of identified specimens or NISP=445, where a specimen is a bone, tooth, or fragment thereof) (Wolverton 2008), determined by the frequency of the most commonly occurring skeletal element, the right femora (where a skeletal element is an anatomically complete specimen). This is because no individual can possess more than one right femur, and since there are 10 right femora there can be no fewer than 10 individual black bears present in this collection. Wolverton (1996, 2008) demonstrated that the site's ursid population is not a representative demographic sample of the biocoenose (the living biological population of ursids at the times when faunal materials were deposited). The site is notably dominated by young adult males (Wolverton and Lyman 1998). This overabundance of young adult males in the taphocoenose (in this case the collection of roughly contemporaneous faunal materials as it existed following the animals' burial, up until their excavation between 1947 and 1959) was likely caused by two factors: the physical structure of the cave, and black bear foraging/dispersal behavior. The cave is a bottle shaped natural trap, with a 178 cm by 79 cm opening and an 11.47 m drop to the cave's bottom (Figures 10 and 11). The entrance to the cave belies its sheer fall. Wolverton (2008) argued that young male black bears are particularly prone to accidental death and the accumulation of bears in

Lawson Cave was likely a result of foraging by young males who had recently dispersed to the area while establishing their own home range, and were thus ignorant of the landscape.

Black bear ecology and social structure helps explain the overabundance of males in Lawson Cave. Black bears are opportunistic omnivores, feeding primarily on vegetation and carrion (Larivière 2001). Their quest for carrion is likely complicit in their falling death into Lawson Cave, which given the occurrence of fauna excavated from the cave floor likely contained considerable amounts of carrion (Wolverton 1996). Black bears live primarily in forested and forest edge habitat, but are capable of living at low population densities in a wide range of habitats. Their mating system is polygynous, with primarily male-based dispersal (Larivière 2001).

In addition to the previously published radiocarbon dates, the occurrence of domestic pig (*Sus scrofa*) (NISP=170) interspersed in the Lawson Cave site suggests that the age of the fauna post-dates the arrival of Europeans (post - AD 1541), since Europeans introduced the domestic pig to Missouri.

Thesis Structure

The next three chapters present the results of a study that collected and analyzed genetic material from the Lawson Cave bear fauna. Chapter 2 details the methods used to prepare and analyze the fauna's ancient DNA. It also describes the site from which the materials were collected and the manner in which samples were prepared for fluorine analysis and radiocarbon dating. Chapter 3 reports the results of this study. It includes the results of ancient DNA as well as comparisons to a modern sample from southern

Missouri and informative genetic sequences gleaned from other similar studies that are publicly available through GenBank. It also reports the results of the fluorine analysis – particularly as it relates to the success and failure of genetic analysis. Chapter 4 analyzes and discusses the results of this study. It includes an appraisal of the decision to repopulate Arkansas with Manitoba/Minnesota black bears in the 1960s. It evaluates whether modern Missouri black bears are genetically distinct from historical black bears in Missouri for the genetic section of mtDNA used in this study. It also situates Missouri black bears in the larger biogeographic map of central North American black bears.

-CHAPTER TWO-

MATERIALS AND METHODS

Sample Preparation

The Lawson Cave faunal assemblage contains 445 skeletal specimens identified as North American black bear (*Ursus americanus*) (NISP=445) (Wolverton and Lyman 1998). Of these specimens, the most commonly occurring element is the right femur (Wolverton 2008). The number of the most commonly occurring elements (n=10) may or may not characterize the total number of individuals in the assemblage (Lyman 2008). There are a number of problems with calculating the minimum number of individuals (MNI) as a measure of abundance (Lyman 1994, 2008); however, in this case, MNI is a valuable metric given concerns about sampling particular individuals multiple times. Since all matrilineally related individuals have identical mtDNA haplotypes, mtDNA cannot be used to identify individuals. Therefore, MNI is used analytically to represent the maximum number of samples that can be analyzed from a population with an unknown total number of individuals without analyzing any particular individual more than once. Without this control 102 samples in which 100 samples belonged to Haplotype α and 2 samples belong to Haplotype β could mean that Haplotype α outnumbers Haplotype β at a rate of 50:1, or since the 100 samples of Haplotype α could be from the same individual it could mean that Haplotype β outnumbers Haplotype α at a rate of 2:1.

Each of the 10 femora were prepared for DNA extraction, fluorine analysis, and AMS dating in the same manner. Three samples were collected from each bone, one for DNA extraction, another for fluorine analysis and one for accelerated mass spectrometry (AMS) radiocarbon-dating (C^{14}). First, a thin (~1 mm) section of the cortex was removed

using a single-use stainless steel razor blade. Then the region where the sample was to be collected was cleaned externally with a 10% bleach solution to remove exogenous DNA that may have been present through handling or storage with other samples (Kalmár et al. 2000). Next ~1 gram of bone was removed using a tungsten cutting bit attached to a Dremel set on low-speed. Care was taken between each sample to clean the Dremel, cutting surface, and bit with 10% bleach solution (Yang et al. 2005). The bit was additionally cleaned with soap, 30% EDTA, and exposed overnight to UV. The samples were then stored in 30ml tubes in preparation for analysis. The manner in which the bone was removed with the cutting bit effectively turned the cortical bone to dust, which obviated any need for further grinding with a freezer mill or mortar and pestle. The only difference between samples prepared for ancient DNA extraction, and fluorine analysis and AMS-dating is that the samples prepared for fluorine analysis and AMS-dating were not cleaned with bleach and the samples prepared for AMS-dating were not turned into bone dust, rather a small complete section of cortical bone was removed. Kemp and Smith (2005) have suggested immersing the entire bone in a 10% bleach solution. This technique, however, was avoided for fear that soaking the bones in bleach would detrimentally affect the fluorine analysis.

Ancient DNA Analysis

DNA Extraction

The DNA extraction was carried out in a room where no ancient DNA analysis had been previously performed and was separated by distance from any other carnivore DNA analysis. The room was thoroughly cleaned with *Eliminase* ™ DNA

decontaminant. All plastic materials (filter tips and tubes) were single use, glassware was cleaned with *Eliminase* ™ between uses and autoclaved frequently, and all chemicals, buffers and reagents were new. Samples were transported in sealed boxes when they left the extraction room (Mulligan 2005). Special care was taken to avoid any cross-contamination at a level commensurate with the risk of contamination. Overall, the risk of contamination is low, given that these samples are neither human nor domesticated (Gilbert et al. 2005). Human and domesticated animal extractions provide a major difficulty in ancient DNA studies, since the analyst, and all the other people who have handled the materials contributed amplifiable DNA, all while the sample continued to lose amplifiable DNA template as a result of the natural processes of DNA degradation.

The DNA was extracted using a modified version of Rohland and Hofreiter's (2007) protocol for extracting ancient DNA from teeth and bones. The protocol was originally written for 500 mg of bone dust, but was scaled down to 250 mg. Five milliliters of extraction solution (0.45 M EDTA and 0.25 mg ml^{-1} proteinase K) were added to the bone dust in a 50 ml tube. This was used to digest the bone matrix and allow the cells to lyse and ultimately release DNA. A negative blank extraction was also included. This sample contained only 5 ml of extraction solution and was used to monitor contamination. All of the succeeding steps were carried out in exactly the same manner with the negative blank extraction.

The tubes were capped and allowed to incubate overnight at room temperature (~16-24 hours) with gentle agitation. After this period of incubation the samples were allowed to settle for ~3-5 hours. The top 3 ml of supernatant were removed with a pipette and transferred to a second tube. The final 2 ml of solution were centrifuged for 2

minutes at 5000 g. The remaining supernatant was removed with a pipette and added to the same second tube.

A chaotropic agent (in this case Guanidine thiocyanate (GuSCN)) was used to bind the nucleic acids in the supernatant to silica (Boom et al. 1990). Twenty milliliters of binding buffer (5 M GuSCN, 25 mM NaCl and 50mM Tris) were added to the second tube, as well as 100 µl of silica suspension (see Rohland and Hofreiter 2007). The solution was adjusted to pH 4.0 by adding ~150 µl of 30% w/v HCl. The tubes were then incubated for 3 hours on a shaker plate. Samples were then allowed to settle for ~2-3 hours. All but 2 ml of supernatant were pipetted out. The final 2 ml were centrifuged for 2 minutes at 5000 g. An additional 1 ml of binding buffer was added to the resulting silica pellet and resuspended. The solution was then centrifuged for 15 seconds at 16000 g.

The supernatant was removed by pipetting. One milliliter of wash buffer (50% v/v ethanol, 125 mM NaCl, 10 mM Tris and 1 mM EDTA, pH 8.0) was added and the silica pellet was resuspended. The solution was then centrifuged for 15 seconds at 16000 g. The supernatant was removed by pipetting. This step was then repeated. The silica pellet was then allowed to dry at room temperature for ~15 minutes with an open lid. To elute the DNA, 50 µl of TE buffer (10 mM Tris, 1 mM EDTA) was added and the silica was resuspended by pipetting up and down. This solution was allowed to incubate with a closed lid for ~10 minutes. The solution was regularly shaken gently. It was then centrifuged for 2 minutes at 16000 g. The supernatant was transferred to a clean tube for PCR amplification. An additional 50 µl of TE buffer were added to the final silica pellet and the final steps were repeated.

DNA Amplification

Primers were designed to amplify the control region of the mitochondria and were specific to *Ursus americanus*. The control region was chosen for three reasons. First, mitochondrial DNA is multicopy and maternally inherited, meaning that multiple copies of the mitochondrial genome are present in every cell. This is important, given the nature of DNA degradation over time. Because DNA degrades over time, primarily through the processes of oxidation and hydrolysis, it is valuable to have a DNA template in large quantities in order to capture contiguous strands of DNA for PCR amplification (Willerslev and Cooper 2005). Second, the mitochondria of animals has a higher substitution rate than single-copy nuclear DNA (5-10 times faster), and the control region is particularly quick to accumulate mutations (4-5 times faster than the rest of the mitochondria) (Taberlet 1996). Because of the speed at which this section changes, the control region is expected to display a higher proportion of polymorphic sites. Since the control region is believed to be a non-coding segment of DNA, meaning that it does not code for any proteins or structural RNA, it has few functional constraints, other than its ability to regulate and initiate replication, and can therefore be expected to evolve in a neutral manner (Irwin et al. 2009). This fact is valuable when using mitochondrial DNA to distinguish intra-specific diversity, since single nucleotide polymorphisms are a valuable tool for distinguishing among groups. The third reason the control region was used in this study is that there is precedent for using it in studies of bears (Barnes et al. 2002; Cronin et al. 1991; Hofreiter et al. 2004; Hofreiter et al. 2005; Pagès et al. 2008; Stone and Cook 2000; Wooding and Ward 1997). Several studies examining both ancient

and modern DNA have used the control region, which makes it an ideal candidate for analysis since it ultimately allowed me to compare the sample black bears with sequences from other studies. A review of the literature turned up no examples of mitochondrial recombination in the ursid control region.

Quantitation

Prior to amplification the quantity of double stranded nucleic acids was evaluated using an Eppendorf BioSpectrophotometer. Since there were multiple elutions of each sample, this allowed me to determine the most suitable elutions to amplify from each. It also allowed samples to be excluded from analysis since the absence of nucleic acids meant that PCR amplification would, at best, be pointless and at worst lead to incorrect conclusions from chimeric or contaminated results (see Hofreiter et al. 2001 for a discussion of using real-time PCR quantification to evaluate this).

Spectrophotometry works by measuring the absorption of UV light. DNA and RNA absorb UV light at particular spectra (230, 260, 280 and 320 nm). The amount of light absorbed is directly proportional to the concentration of total nucleic acid. However, since these samples were collected from specimens that were at various times in a cave, in a museum, and handled by multiple individuals it is not only possible, but indeed likely that these samples contained nucleic acids that were not part of the specimen of interest. Therefore, the amount of nucleic acid in each sample, determined by spectrophotometry, cannot be assumed to be an accurate measure of *Ursus americanus* DNA in the sample. Spectrophotometry allowed for the exclusion of 3 samples (in which every elution contained no nucleic acids), but included 3 samples that I was not able to

amplify. The 3 samples that contained nucleic acids but failed to amplify may have contained nucleic acids from other sources or simply been too fragmented from hydrological leaching and oxidation to amplify.

Primers

Primers were designed using *Primer3* (Rozen and Skaletsky 2000 http://frodo.wi.mit.edu/). Three pairs of primers (5F/5R, 6F/6R, and 7F/7R; Table 1) were selected in order to amplify and sequence 351 base pairs of the control region of the mtDNA (Figure 12). Ultimately two of the primers needed to be redesigned (5R and 6F) to allow the amplification of certain samples. The redesigned primers are designated 5F2 and 6R2 (Table 1).

Primers were selected to amplify small overlapping sections of mtDNA. This is because of the general expectation that aDNA is degraded and contains only small DNA fragments (Hofreiter et al. 2001). Therefore, rather than using primers designed to amplify the entire range of the sequence in question, smaller sections of sequence (product size < 100) were entered into the *Primer3* input. These were then aligned following sequencing.

Polymerase Chain Reaction

PCRs were performed in 25 µL reaction volumes containing 1x PCR Gold buffer (Applied Biosystems), 2 mM dNTP, .4 mM each of forward and reverse primer, 2 mM $MgCl_2$, .8x BSA, Ampli*taq* Gold DNA polymerase 1U (Applied Biosystems), and 2-4 µL of template DNA. PCRs were run in the following thermal cycling conditions: 95 ºC

denaturation for 10 minutes, followed by 45 cycles of 1 minute denaturation at 95 ºC, 1 minute primer annealing at between 53 ºC and 59 ºC, and 1 minute primer extension at 72 ºC, and a final primer extension step of 10 minutes at 72 ºC. All PCRs included negative controls for both the PCR and extraction steps in order to detect possible contamination. The results of the PCRs were confirmed with gel electrophoresis (2% agarose).

Cloning

Fragment 6F and 6R2 for sample Ua5 was cloned using a Topo TA Cloning Kit (Invitrogen) and was prepared for sequencing using a QiaPrep Spin Multiprep Kit (Qiagen). Ten separate colonies were prepped to obtain confirmatory sequences. This was done because only one elution of Ua5 successfully amplified by PCR for fragment 6F and 6R2. The PCR was then repeated and the products were cloned in order to achieve replicated results, a necessity for studies of ancient DNA, given concerns about contamination and false results.

Sequencing

PCR products were purified for sequencing using a QiaQuick Spin Column PCR Purification Kit. Two to four μL of clean PCR product were added individually to 12-14 μL of 5pM forward or reverse primer. These reactions were sequenced at the the University of Missouri DNA Core Facility in an Applied Biosystems 3730 DNA Analyzer with Applied Biosystems Big Dye Terminator cycle sequencing chemistry.

Data Analysis

Sequences were compared to GenBank samples using a nucleotide Basic Local Alignment Search Tool (BLAST) search to confirm that they were from *Ursus americanus*. They were then aligned using SEQUENCHER 4.5 (GeneCodes Corporation). The three samples from this project were combined with 28 samples from a prior study of modern black bears in southern Missouri as well as sequences gleaned from GenBank (Onorato et al. 2004; Van Den Bussche et al. 2009). The model selected for this analysis was the HKY model, which was determined using MODELTEST 3.7 (Posada and Crandall 1998). Phylogenetic analyses were run using PAUP* 4.0. Asiatic black bear (*Ursus thibetanus* (GenBank Accession: NC009971)) and sun bear (*Helarctos malayanus* (GenBank Accession: EF196664)) sequences were selected as outgroups. The outgroups were chosen because several ursid mitochondrial phylogenies have determined *Ursus thibetanus* and *Helarctos malayanus* as sister taxa to *Ursus americanus* (Bon et al. 2008; Krause et al. 2008; Yu et al. 2004). Neighbor Joining, UPGMA, Maximum Parsimony, and Maximum Likelihood (based on the MODELTEST 3.7 parameters) phylogenies were run for all sequences, and produced the same general topology. Trees were selected on the basis of their bootstrap support. The Neighbor Joining method was selected for the two phylogenies used in this thesis.

Dating

Fluorine Analysis

Samples from all specimens were sent to the University of Missouri Research Reactor for fluorine analysis. There the samples were prepared for neutron activation analysis (NAA). The results of the analysis were reported in terms of sodium (^{23}Na) corrected fluorine (^{20}F) average concentrations (ppm) (Table 2).

AMS Radiocarbon Dating

Based on the results of the fluorine analysis 3 specimens were sent to the NSF Arizona AMS Facility for Accelerate Mass Spectrometry Radiocarbon Dating (reference: AA84746, AA84747, and AA84748). These included samples Ua2, Ua5 and Ua8 (Table 2). Samples were selected on the basis that they include the upper and lower range of sodium-corrected fluorine values (Ua2 and Ua8) as well as an intermediate value (Ua5), to provide a test for a later linear regression analysis of the fluorine and AMS values.

-CHAPTER THREE-

RESULTS

In this chapter I present the results of my genetic analysis. In order to situate the 10 *Ursus americanus* right femora from Lawson Cave in the history of North American black bears I include a phylogenetic analysis of mtDNA from samples collected from southern MO and mtDNA sequences gathered from GenBank. In addition to the genetic results, I also present results of the fluorine analysis and radiocarbon dating, both aimed at determining the specific chronology of genetic events as well as the appropriateness of treating all four samples as contemporaneous.

AMS Dates

The three samples sent to the NSF Arizona AMS Facility came back with dates ranging from 206±41 (BP) to 630±42 (BP) (Table 2). Ua2 and Ua5 returned roughly contemporaneous dates. Ua8 was unamplifiable and returned a date that is non-contemporaneous with the other samples (Table 2).

Ua2 and Ua5 have similar ^{20}F values and also have similar C^{14} dates. Ua8 has a much higher ^{20}F value and is subsequently much older. Taken together these data suggest that it is justifiable to use ^{20}F values as a measure of relative chronology (McConnell 1962; Lal 1975). It also suggests that the entire group of amplified sequences can be treated as contemporaneous since they have similar ^{20}F values. This analysis places the amplified sequences (Ua1, Ua2, Ua4, and Ua5) firmly in the Historical Era (0-550bp) and confirms that these sequences co-occur with arrival of European settlers, but occur before the massive deforestation events of the late 19th and early 20th centuries. These samples,

therefore, provide a valuable historical snapshot of Missouri's most recent unambiguously native black bear population.

Sample Success

Each of the ten *Ursus americanus* right femora from Lawson Cave (involving multiple extractions and elutions from each individual specimen) was subjected to the same extraction rigor. They were subsequently analyzed with a spectrophotometer and if nucleic acids were detected, 2-5µl of sample were amplified by PCR and then checked with gel electrophoresis. Amplification products from sample Ua5 were cloned, in order to achieve confirmatory results. As a result, four of the 10 samples were successfully sequenced using this methodology (Table 2).

The results of fluorine analysis indicate that there is a trend between the concentrations of Na corrected ^{20}F and the success or failure of genetic amplification (Table 2). Samples with the highest fluorine values did not amplify by PCR, and the four samples with the lowest fluorine values did amplify. This result helps validate this study's methodological rigor and suggests that the failure of six of the extractions to amplify was not due to improper labwork or a lack of effort, but rather the initial conditions of the individual sample's genetic material.

Fluorine enrichment in fossilizing bone is primarily the result of the chemical conversion of bone's hydroxyapatite ($Ca_5(PO_4)_3OH$) into the more chemically stable fluoroapatite ($Ca_5(PO_4)_3F$) (Gaschen et al. 2008). The primary mechanism believed to be responsible for this exchange of chemicals is the percolation of groundwater into the pores of bones, either through the medullary cavity or into the Haversian canals (Gaschen

et al. 2008; Hedges and Millard 1995; Reich et al. 2002). This process is exacerbated in acidic environments and as a result of microbial intrusions (Coote 1992).

In non-permafrost conditions the cleavage of DNA's phosphate sugar backbone, resulting in single strand breaks, is believed to be the dominant type of damage in ancient DNA (Mitchell et al. 2005). This damage occurs as a direct result of base loss caused by hydrolysis (Lindahl 1996). DNA hydrolysis occurs because of the presence of water and results in the chain rupture at abasic sites (Lindahl 1996).

Since fluorine analysis is an indicator of groundwater uptake, it stands to reason that it is also a derived measure of hydrolysis. Although this relationship remains to be tested and established in terms of a known set of chemical reactions, the correspondence between amplification success and fluorine concentration suggests that absolute fluorine concentration may be a useful measure of DNA damage – base pair hydrolosis.

Sequence Alignment

Sequence alignment of six fragments for each sample using SEQUENCHER 4.5 yielded four contiguous 351 base pair sequences (Figure 13). Based on this section of the control region of the mitochondria two of the four samples are genetically indistinguishable, meaning that two of these samples are genetically identical in terms of the section of sequence used in this study (Ua1 and Ua4). Sample Ua2 has five single nucleotide polymorphisms that distinguish it from all other samples. Sample Ua5 has four single nucleotide polymorphisms and a base pair deletion that distinguish it from all other samples. Ua5 and Ua2 do not share any nucleotide polymorphisms that distinguish them from Ua1 and Ua4. These four samples therefore, yield three distinct mitochondrial

haplotypes based on this sequence alignment. Replicated measures, either through the sequencing of multiple elutions, or through cloning confirmed the results of this sequence alignment, and in cases of ambiguity at least three independent samples were sequenced, aligned, and analyzed. This methodology helps support the validity of these results and minimizes concerns about the well known problems related to artifactual base pair transitions, particularly cytosine deamination, which is a process whereby individual cytosine nucleotides are converted to uracil through oxidative damage, and are subsequently read as thyamine after PCR (Hofreiter et al. 2001b; Lindahl 1993). Most of the transitions among Ua2, Ua5 and Ua1 and Ua4 (collectively) are of cytosine to thymine transitions (or vice-versa). Without controlled and repeated measures there could be only minimal confidence in the results of this study. However, since cytosine deamination affects particular molecules, and not the overall template, repeated measures provide a powerful confirmatory test.

BLAST Results

Following the sequence alignment, the Lawson Cave sample sequences were entered into GenBank's nucleotide BLAST search. GenBank's BLAST search looks through its massive collection of DNA sequences to determine which of the sequences in its database most closely resemble the sample sequences. The results of this search are then reported in terms of percent homology. In this case homology simply refers to exact nucleotide-to-nucleotide matches, and not to inferences about the samples' evolutionary history. Using a BLAST search samples Ua1 and Ua4 were a perfect (100% homology) match for *Ursus americanus* haplotype B (GenBank Accession AY334364) (Onorato et

al. 2004). Samples Ua5 and Ua2 have no perfect (100%) haplotype homologs in the GenBank database.

It is potentially informative, however, to point out that haplotype B extends 264 base pairs on the 3' end and samples Ua1 and Ua4 extend 48 base pairs on the 5' end. The sequences, therefore, are not totally comparable, even though they overlap by 303 base pairs (Table 5.1 and Table 5.2). In terms of polymorphic sites, when comparing this study's samples with published haplotypes A-M (GenBank Accession AY334363-AY224367; FJ619652-FJ619659) (Onorato et al. 2004; Van Den Bussche 2009), there are four informative polymorphic sites on the 3' end that have not been sequenced for the Lawson Cave samples and four potentially informative polymorphic sites on the 5' end that were not sequenced by the authors of the GenBank samples (Onorato et al. 2004; Van Den Bussche et al. 2009) (Tables 5.1 and 5.2). The region where samples Ua1 and Ua4 and haplotypes A-M overlap is, however, sufficiently polymorphic to separate the Lawson Cave samples from all known *Ursus americanus* haplotypes except haplotype B. This gives strong support for the statement that if samples Ua1 and Ua4 are members of any of the haplotypes reported to GenBank, they are members of Haplotype B.

Phylogenetic Analysis

Phylogenetic analysis in this case allows us to determine the nearness of genetic relationships between the individual bears represented by the samples, and to hypothesize about the evolutionary history of *Ursus americanus* in central North America. It is inappropriate to generate a phylogenetic tree based solely on the four successfully sampled individuals, since there were only three haplotypes present and the phylogeny

would be meaningless in light of any larger historical insights. Therefore this phylogenetic analysis includes samples collected by Faries and Eggert in 2008 from southern Missouri, samples submitted to the Eggert Lab for mtDNA sequencing from Minnesota and Arkansas, as well as haplotype sequences obtained from the GenBank database (GenBank Accession AY334363-AY224367; FJ619652-FJ619659). These samples and sequences collapse into 16 distinct mitochondrial haplotypes, hereafter referred to as haplotypes A-P following Van Den Bussche et al.'s (2009) determination of 13 haplotypes covering central North America from Manitoba to north-central Mexico, as well as the 3 distinct mtDNA sequences identified from the samples submitted to the Eggert Lab.

The three ancient DNA sequence haplotypes were excluded from the primary mtDNA phylogeny. This is because creating a phylogenetic tree from disparate sequence lengths and coverage areas requires us to crop the sequence to equal portions in the same coverage area. The result of this is a loss of potentially informative data. Including the ancient haplotype sequences would require us to omit 4 of the polymorphic sites on the 3' end, which amounts to 18.18% of the total number of polymorphic sites. This is problematic because although these sites are unnecessary in determining the identity of Ua1 and Ua4 within haplotype B, or in distinguishing Ua2 and Ua5 from all known haplotypes, they are potentially valuable in resolving the species' phylogenetic history.

Because the sequence regions used in this study did not cover identical areas, the strategy used to create a phylogenetic tree in this study was two-fold. First, I created a phylogenetic tree using PAUP* for haplotypes A-P which includes the entire section of the control region contained in each GenBank sequence. The purpose of this phylogenetic

tree is to display the phylogenetic history for *Ursus americanus* throughout central North America. Second, I cropped all the sequences to the length and coverage area of the ancient samples. This second phylogenetic tree is meant to visualize the phylogenetic history of a particular clade of central North American *Ursus americanus* haplotypes, including the sequences collected from Lawson Cave.

The phylogenetic tree in Figure 14 shows two distinct clades, one that includes haplotypes {H, F, C, P, B, A, K, and G}, hereafter referred to as clade 1, and one that includes the other haplotypes {D, M, O, I, N, E, L, and J}, hereafter referred to as clade 2. Bootstrap values for clade 1 (71%) show that it is distinct and fairly well supported. Clade 2 is less well supported, but is phylogenetically distinct from clade 1.

Van Den Bussche et al. (2009) and Onorato et al. (2005) report the occurrence of haplotypes A-M in geographic space. Table 3 shows the regions in which each haplotype is present. Missouri samples primarily occur in clade 1. Both clades are pan-continental throughout the central region of North America. Most of the geographic variability in clades 1 and 2 is the result of the ranges of haplotypes B and E (Table 4) (Figures 15 and 16).

After cropping the sequences to the length and coverage area of the ancient samples, the primary tree topology based on longer sequences is preserved (Figure 17). But, the loss of sequence data includes a concomitant loss in phylogenetic precision, particularly in terms of distinguishing the phylogenetic relationships in clade 2. Even with this loss of precision, bootstrap values remain significant for clade 1 (71%). Of primary importance in this study is that samples Ua2 and Ua5 group with clade 1. Since

samples Ua1 and Ua4 belong to haplotype B, all 4 of the Lawson Cave samples can be effectively grouped into clade 1.

Missouri Sample Assignment

Including the ancient DNA sequences from Lawson Cave, 23 Missouri samples were included in this analysis (four individuals from Lawson Cave and 19 modern samples). Four of these samples (17%) fall into haplotype B (Figure 18). These samples include individuals from Nixa, MO, Oregon County, MO, and Lawson Cave, MO. Fifteen of the samples (65%) fall into haplotype E. The majority of these individuals (10 out of 15) were collected from Seymour, MO; the rest were collected from Jadwin, MO, Rover, MO, Caney Mountain, MO, Brushy Creek, MO, and Miley, MO. One individual falls into Haplotype A. The sample from this individual was collected in 1984 from Reynolds County, MO. In addition 3 samples had no perfect (100% BLAST sequence similarity) matches to any known haplotype. These 3 unique haplotypes include one individual from Ozark County, MO, and two individuals from Lawson Cave.

Eight of the 23 samples (35%) from Missouri fall into clade 1. These 8 include the 4 samples from Lawson Cave as well as the other individuals from Haplotypes B and A. Excluding the Lawson Cave samples, only 32% of the Missouri samples come from clade 1. The other 68% come from clade 2. All of the individuals that can be grouped with clade 2 are members of Haplotype E. The historic distribution of this haplotype is unknown, however, it currently occurs in Minnesota, Manitoba, Arkansas, and seemingly disjointedly in the Mogollon Mountains of New Mexico (Onorato et al. 2007).

Van Den Bussche et al. (2009) report that as with modern Missouri black bears, modern Arkansas black bears fall primarily into haplotypes B and E. The exception to this statement is the black bear population from the White River Natural Wildlife Refuge that falls exclusively into haplotype B. This group of bears is believed to be a relict population that survived the massive deforestation events of the late 19th and early 20th century.

In the final chapter I analyze these results in an attempt to describe Missouri's place in the historical phylogeography of black bears in central North America. I also offer an explanation for the current diversity of haplotypes in the modern Missouri black bear population. Finally, I evaluate the historic translocation of black bears from Manitoba and Minnesota into Arkansas and the impact that it has had on the modern black bear population in Missouri, particularly as it relates to the historical black bear population.

-CHAPTER FOUR-

DISCUSSION

This study was initiated with the goal of determining what contribution the translocation of black bears from Minnesota and Manitoba into Arkansas had on the genetic constitution of the modern population of black bears in southern Missouri. Given the severe decimation of the regional black bear population in the late 19th and early 20th century as the Missouri hardwood forests shrunk to 2% their original size, it is unlikely that the Missouri population could have rebounded to a robust size with a healthy amount of genetic diversity. It is not, however, impossible that Missouri bears could have contributed to the genetic makeup of the modern growing population. The genetics of Lawson Cave bears provide an empirical historical basis for evaluating claims about whether or not the modern population is *analogous* to the population of black bears that historically occupied Missouri. By *analogous* I mean that the modern population is genetically indistinguishable from the historical population. Similarity by *analogy* is distinct from similarity through *continuity* in that the former only makes a claim about our ability to genetically differentiate between the two populations, whereas the latter requires the demonstration that genetic similarity is due to the unbroken inheritance of haplotypes *in situ*.

One of the facts that became evident as analysis proceeded was that the control region of the mitochondria does not define a single bounded Missouri or mid-central United States black bear haplotype. It is possible that microsatellite markers will provide more fine-scale subpopulational dynamics and signatures from this large contiguous region, and in fact current research by Eggert and Faries (pers. comm.) is attempting just

such a study. For this geographic area, however, the control region of the mitochondrion is not sufficiently polymorphic to allow Missouri, or even Missouri and its immediately surrounding states to be genetically differentiated from the rest of North America.

The reasons that using the control region to distinguish a particular signature for Missouri's native black bear population produces such complicated results are two. First, two dominant haplotypes found among Missouri black bears in this study (both from Lawson Cave and from the modern southern Missouri population) are haplotypes B and E. An individual of haplotype A was found in Reynolds County, MO, in 1984, but only one individual of this haplotype has been found. Two historic and two modern individuals both fall within haplotype B. The rest of the individuals (n=15) belong to haplotype E.

Haplotype B is a pancontinental haplotype extending from Minnesota through north central Mexico and includes the modern populations in the Ozark National Forest in Arkansas, Ouachita State Parks in Oklahoma and Arkansas, the White River Natural Wildlife Refuge in southern Arkansas, sites in Louisiana and sites in Texas (Van den Bussche 2009). Haplotype B is found in a roughly continuous distribution through most sites that have been evaluated in central North America south of Minnesota. Even though several of the populations in Arkansas, Texas, and Louisiana are known to have received translocated individuals, the presence of haplotype B in White River Natural Wildlife Refuge, Tensas Louisiana, Coastal Louisiana, and, Mexico, as well as in Lawson Cave points very strongly to the historically continuous distribution of this haplotype throughout this region.

The dispersal pattern and home range characteristics of black bears suggest a reason for this wide distribution. Although empirical evidence for female-based dispersal in mammals has mounted considerably in the last three decades, male-based dispersal remains the model for mammalian sex-based dispersal (Greenwood 1980; Handley and Perrin 2007). Black bears follow the common pattern in mammals whereby females establish a home range relatively near their natal area, while males disperse to areas outside of their natal area (Larivière 2001).

Kemp's (1976) study of black bear in northern Alberta found that 70% of males dispersed more than 4 km outside of their natal area. Payne's (1975) study in British Columbia found males dispersing as far as 179 km. Ruthglen and Herbison's (1977) study of black bears in Newfoundland found males that had dispersed 99 km. Schwartz and Franzmann (1992) found that 100% of the males in Kenai Peninsula dispersed outside of their natal area. Costello et al. (2008) found that all 290 male black bears they studied in New Mexico had dispersed more than 20 km each. Cronin et al. (1991) did a genetic study of several species of bear in northern North America. Their study utilized RFLPs (Restricted Fragment Length Polymorphism), a genetic technique for determining polymorphic regions of DNA. Cronin et al. (1991) found the same haplotypes in far eastern and northwestern North America and suggested that dispersal might be a confounding issue. An additional anecdote is the recovery of a juvenile black bear from a road kill site in Ozark County, MO, during the study by Eggert and Faries in 2007 (pers. comm.). This individual had been tagged in its natal den in Ozark National Forest in Arkansas. The distance from where it was tagged to where it was recovered is more than 200 km.

Because of the particular characteristics of mitochondrial inheritance, the distribution of Haplotype B across such a large geographic space cannot be explained by male dispersal. This is because males are capable of inheriting mitochondria from their mothers and subsequently geographically dispersing it, but they cannot pass this haplotype to the next generation. As such, males provide a mitochondrial dead end.

More informative in this case are the particular characteristics of female black bear home range size. Female black bears are not, strictly speaking, dispersers. They are more appropriately thought of as range expanders. Female home range sizes vary as a result of both geography and ontogeny. Benson and Chamberlain's (2007) study of female black bears in Louisiana found that in the Tensas River area female home ranges averaged 11.9 km^2, while in the Deltic Region it averaged only 3.52 km^2. Garshelis and Pelton (1981) found that female black bear home ranges in the Great Smokey Mountain National Park averaged 15 km^2. Alt el al.'s (1980) study of female black bear home ranges in northeastern Pennsylvania averaged 41 km^2. Reynolds and Beecham's (1980) study of home range sizes for female black bears in central Idaho averaged 12 km^2.

More informative than home range size, however, is home range overlap and adjacency. While female black bears don't disperse in the same way as males, they do move into unoccupied areas to establish their home ranges. Hellgren et al. (2005) found that female black bears moved between 29 and 128 km (averaging 76 km) to establish their home range. Ulrey (2008) found that female black bears in central Florida had an average home range size of 69.0 km^2, but if subadults were excluded this average dropped to 45.1 km^2, suggesting that subadult females move into new (non-maternal) home ranges and then constrict those ranges once they become adults and begin raising

cubs. Shenk et al.'s (1998) study of female black bear home ranges in Ontario, Canada found that 23% of female black bears have non-overlapping and non-adjacent home ranges, 27% have non-overlapping adjacent home ranges, 31% have home ranges with very little overlap, and only 29% have home ranges with a moderate amount of overlap.

Taken together, these studies of home range suggest a pattern of behavior that has a distinct mitochondrial consequence. As female black bears give birth to female cubs that establish relatively expansive home ranges outside of the mothers' home range, the genetic consequence is the geographic expansion of the mitochondrial haplotype. Given the extreme adaptability of black bears to a variety of diets, climates and habitats, the barriers to gene flow should be limited to major geographic barriers such as extremely arid deserts (Lavière 2001), provided we exclude the modern barrier of interstate highways, which appear to have very real consequences in terms of black bear dispersal ability (Dixon et al. 2005). Furthermore, given that the genetic sequence being studied (the control region of the mitochondria) is non-coding and therefore unlikely to be under selective pressure, it is expected that this region will evolve in a neutral manner. Under such conditions the primary mechanisms for maintaining a diversity of haplotypes are not expected to be isolation by distance solely, in which particular haplotypes group together in particular regions of geographic space, but may also include widespread genetic drift (Wright 1943). This pattern would explain the continent-wide distribution of haplotype B, even occurring in the same area as other haplotypes.

The presence of haplotype E presents an opportunity to deal with something of an anomaly. Van Den Bussche et al. (2009) point out that haplotype E is present in the Ozark National Forest, AR, and both Lake Ouachita State Parks in OK and AR. This

haplotype is present in Manitoba and Minnesota, but was also found by Onorato et al. (2007) in the Mogollon Mountains of New Mexico. Therefore the haplotype has a large modern geographic distribution. However, since there has, as of the writing of this paper, been no comparative study of mitochondrial haplogroups extending north from New Mexico along the Rocky Mountains, it is unclear whether the true distribution of this haplotype runs parallel to haplotype B. It is also possible that the presence of haplotype E in the Mogollon Mountains is the result of a continuous geographic population in the area now occupied by haplotype B. This once continuous population may have recently been replaced by haplotype B. And therefore the presence of haplotype E in the Mogollon Mountains may mean that it is a relict population leftover as the result of a large replacement event. It is also possible that it is a mix of both of these scenarios. Ultimately, understanding the occurrence of haplotype E will require a more widespread comparative study that incorporates samples from both western and central North America.

The occurrence of haplotype E in the modern Missouri population therefore warrants some discussion. Without a distinct genetic signature is it possible to exclude haplotype E as a native haplotype? In this case we are forced to contend with the question: Is the absence of evidence for haplotype E in historical Missouri evidence for the absence of this haplotype as native to Missouri? Answering this question requires that we address the second confounding issue in distinguishing a particular genetic signature for Missouri, namely the diversity of the source population.

The current genetic composition of the population of black bears in Missouri is the result of either the native genetic contribution, the repopulation of the area by

immigrants from Arkansas that were translocated from Minnesota and Manitoba, Canada, or both. The problem in evaluating these scenarios is that of all the regions used in this study, Minnesota and Manitoba have the greatest haplotype diversity. It is very likely that the translocated bears repopulated Missouri with a genetically analogous population. It is also likely that they included haplotypes that were distinctly non-native to the region. Since the translocated bears were not selected on the basis of their genetics, and since control region haplotypes have no phenotypic correlates, the mtDNA haplotypes of these bears were likely a random sample from whatever region the conservation agents selected them.

It is possible that haplotype E has simply not *yet* been found in any of the middle central North American sites that were not affected by translocations. In this case however, strong inference suggests that it is not native to the region, and that individuals with haplotype E occur in southern Missouri solely as the result of transplantation by people. There are four lines of evidence supporting this claim.

First, haplotype E is not found among the samples collected from Lawson Cave. While, this sample is not of sufficient size to generate a statistically meaningful representation of the total historical haplotype diversity of the region, it is sufficient to provide a line of evidence for the historical haplotype constitution. Lawson Cave essentially establishes a genetic frame of reference for evaluating native vs. nonnative modern haplotypes. As we extend the number of samples that comprise the historical genetic frame of reference it should be expected that it will provide a more complete and representative sample of the haplotype diversity in the region. Currently, this study has found that there is evidence of haplotype B in the paleozoological record, but not for

haplotype E. If further studies of historical era sites in Missouri find haplotype E deposited naturally in their faunal assemblage, than the conclusion that haplotype E is non-native to Missouri will be effectively invalidated.

The second line of evidence supporting this claim is that all 18 of the black bears sampled at the White River National Wildlife Refuge were members of haplotype B. The presence of haplotype B in this region is significant because this group of presumably native black bears is nearest to the modern population in southern Missouri (Csiki et al. 2003). This fact suggests that black bears in Missouri may have also been members of this haplotype. As telling as the occurrence of haplotype B at the White River National Wildlife Refuge is, equally telling is the noticeable absence of haplotype E in that refuge.

Third, haplotype E occurs in Minnesota and Manitoba, as well as in the Ozarks and Ouachitas of Arkansas and Oklahoma. Thus, a random sample of hundreds of black bears from Minnesota and Manitoba would have likely included individuals of this haplotype. The occurrence of haplotype E in the Ozark and Ouachita regions of Arkansas and Oklahoma makes possible the claim that translocated bears subsequently colonized Missouri. Had this haplotype not occurred in Minnesota or Manitoba it would be impossible to claim that its occurrence was the result of a translocation event. Furthermore, had its presence in modern Arkansas and Oklahoma not been firmly established, then it would be impossible to claim that the presence of this haplotype in modern Missouri was the result of a northern immigration of Arkansas black bears.

Fourth, haplotype E is found discontinuously in central North America. Although it is found in both Minnesota/Manitoba and the Mogollon Mountains of New Mexico, its absence from any known site in between, either historical or modern in the intermediate,

warrants an explanation other than postulating a recent continuous population from Minnesota/Manitoba through Missouri to New Mexico. An explanation for the occurrence of haplotype E in New Mexico may ultimately await studies of mtDNA from black bears along the entire Rocky Mountain region, from Canada through to New Mexico. If further comparative studies find that haplotype E occurs only in western North America, and not in central North America, it would further support the claim that clade 2 occurs in Arkansas, Oklahoma, and Missouri as the result of a recent translocation event.

Although this study has demonstrated that haplotype B represents a historical haplotype in Missouri, the evidence at hand does not allow a determination as to whether the current existence of this haplotype is the result of a temporally continuous population or a replacement event by a genetically similar, but not historically continuous population. The control region of the mitochondria is too imprecise to be useful in settling this argument. What can be said is that there are bears in southern Missouri today that represent the same haplotype as bears that existed in the state historically. Whether this warrants the former being categorized as a *native* population is a completely separate question.

Conclusions

Eggert and Faries (pers. comm.) have found 3 existing haplotypes (A, B, and E) in modern Missouri. Most individuals from their study fall into haplotypes B and E. The historical Lawson Cave population includes individuals from haplotype B, but none from haplotype E. Furthermore, other studies (Onorato et al. 2007; Van Den Bussche et al.

2009) have found haplotype B in a nearby site (White River National Wildlife Refuge) and throughout central North America. Haplotype E, on the other hand, has only been found in Arkansas, Oklahoma, Minnesota, Manitoba and New Mexico. Taken together, these facts support the inference that haplotype B represents a *native* Missourian haplotype. However, given the limits of the region of mitochondrial DNA analyzed in this study, it is impossible to determine whether the presence of this haplotype in Missouri has been temporally continuous for the last several hundred years or whether it is the result of a transplantation event.

Evaluating the translocation of black bears from Minnesota and Manitoba into Arkansas in the 1950s and 1960s, in terms of their impact on the Missouri population, two facts become clear. First, Miller et al.'s (1998) claim that the black bear reintroductions in the 1950s and 1960s had no impact on the modern genetics of the central North American states is unsupported. Given our current knowledge, haplotype E is new to the region and the distinct result of transplantation. Second, there are modern individuals that possess the historical haplotype in Missouri. Whether this is the result of conservation agents' fortuitous decision to select from areas that have proven to be some of the most genetically diverse in North America, or whether this represents the in situ persistence of an ancestral haplotype is unclear. What remains clear is that haplotype B exists in this area both currently and historically.

Figure 1. Historic Era (0-550 bp) Archaeological Sites Containing *Ursus americanus* Faunal Specimens (Reprinted from FAUNMAP). Diamonds indicate the presence of Historical Era sites that contain *Ursus americanus* specimens in their collection. This map is incomplete, and a noticeable omission is Lawson Cave.

Figure 2. Location of Lawson Cave in the State of Missouri (Lat: 38º50'36.50" N, Long: 92º17'05.98").

Map 497. *Ursus americanus.*

1. *U. a. altifrontalis*
2. *U. a. amblyceps*
3. *U. a. americanus*
4. *U. a. californiensis*
5. *U. a. carlottae*
6. *U. a. cinnamomum*
7. *U. a. emmonsii*
8. *U. a. eremicus*
9. *U. a. floridanus*
10. *U. a. hamiltoni*
11. *U. a. kermodei*
12. *U. a. luteolus*
13. *U. a. machetes*
14. *U. a. perniger*
15. *U. a. pugnax*
16. *U. a. vancouveri*

Figure 3. *Ursus americanus* Distribution. Dots represent actual localities where specimens were collected and are used to define the margins of the distribution. "[S]haded areas represent only the author's [Hall] guesses as to the occurrence" (Reprinted from Hall 1981, quote p. viii).

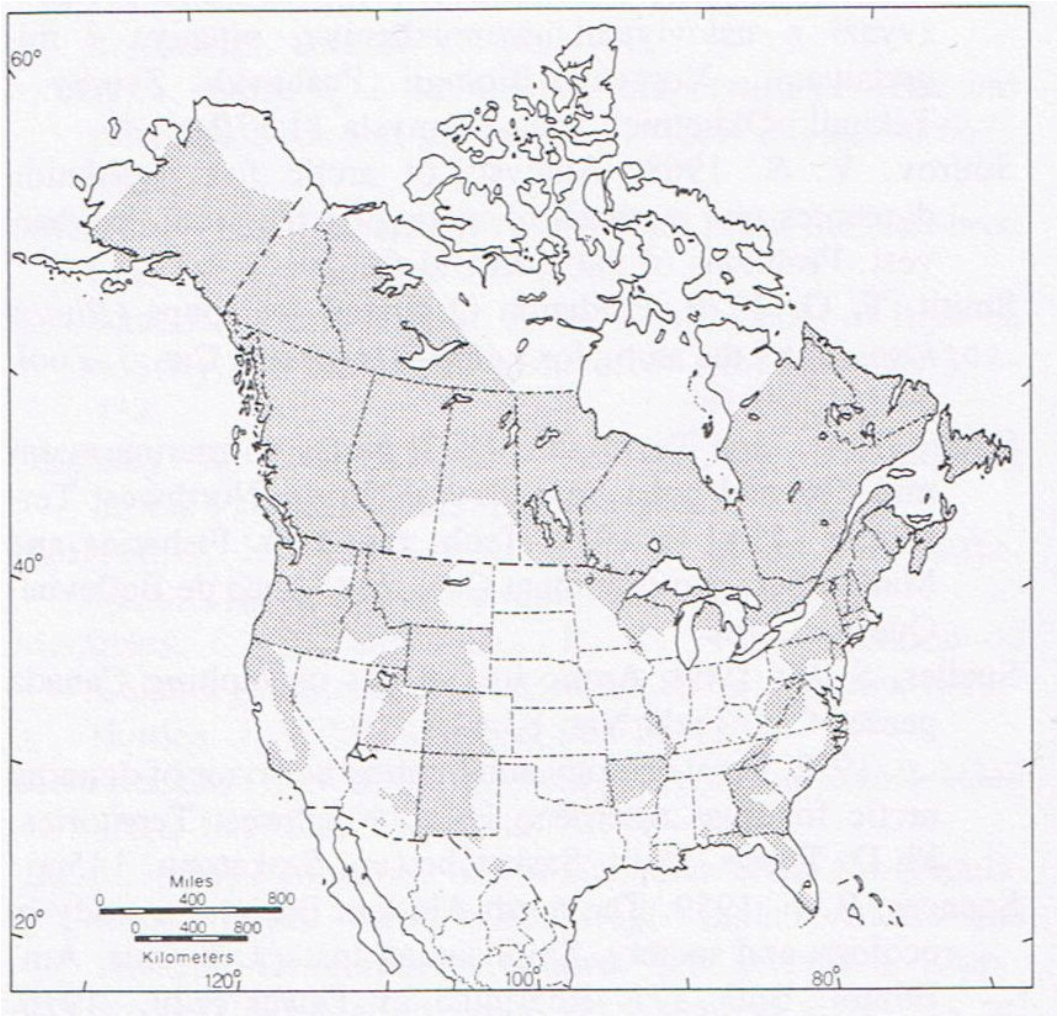

Figure 4. *Ursus americanus* Distribution. This distribution is presumably modern and based on research and conservation reports published before 1982 (Reprinted from Pelton 1982).

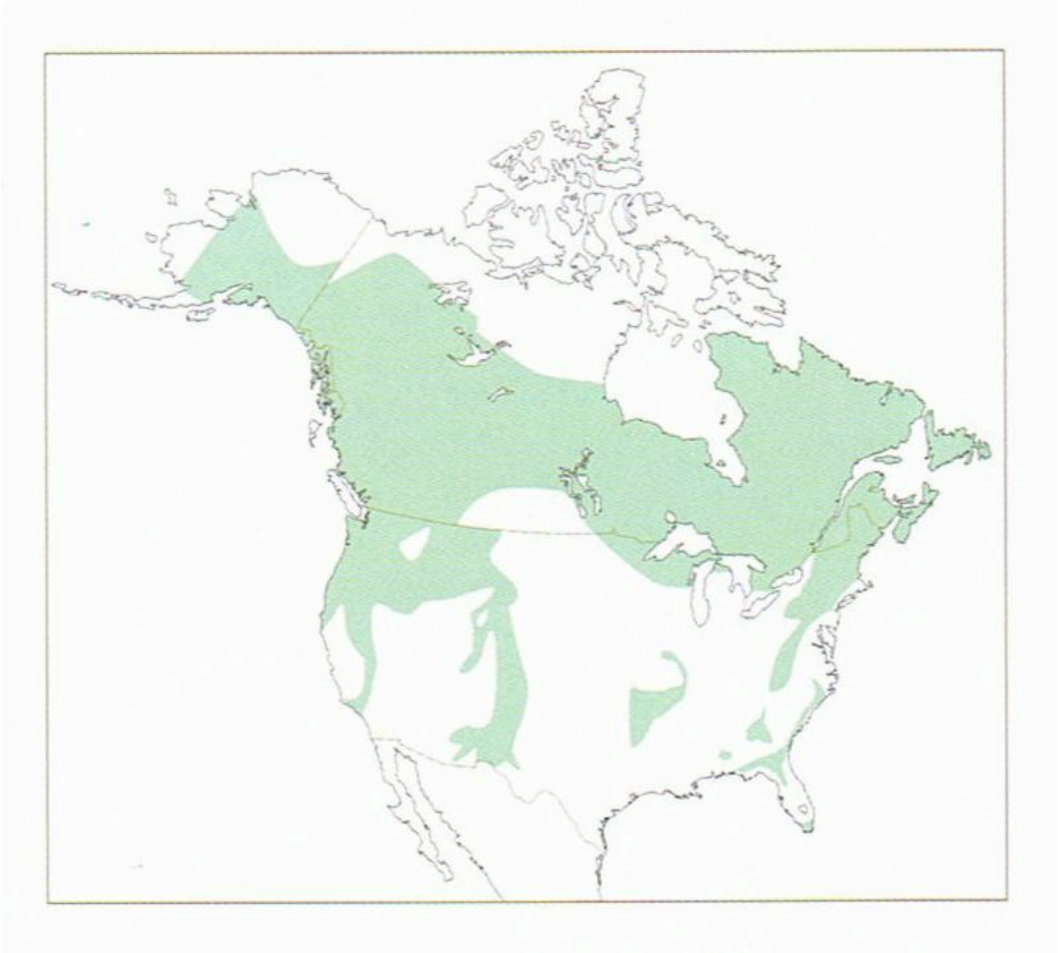

Figure 5. ***Ursus americanus*** **Distribution. Forsyth does not describe the basis for this distributional map. Presumably it is modern and was drawn from the distribution map presented in Pelton (1982) (Reprinted from Forsyth 1999).**

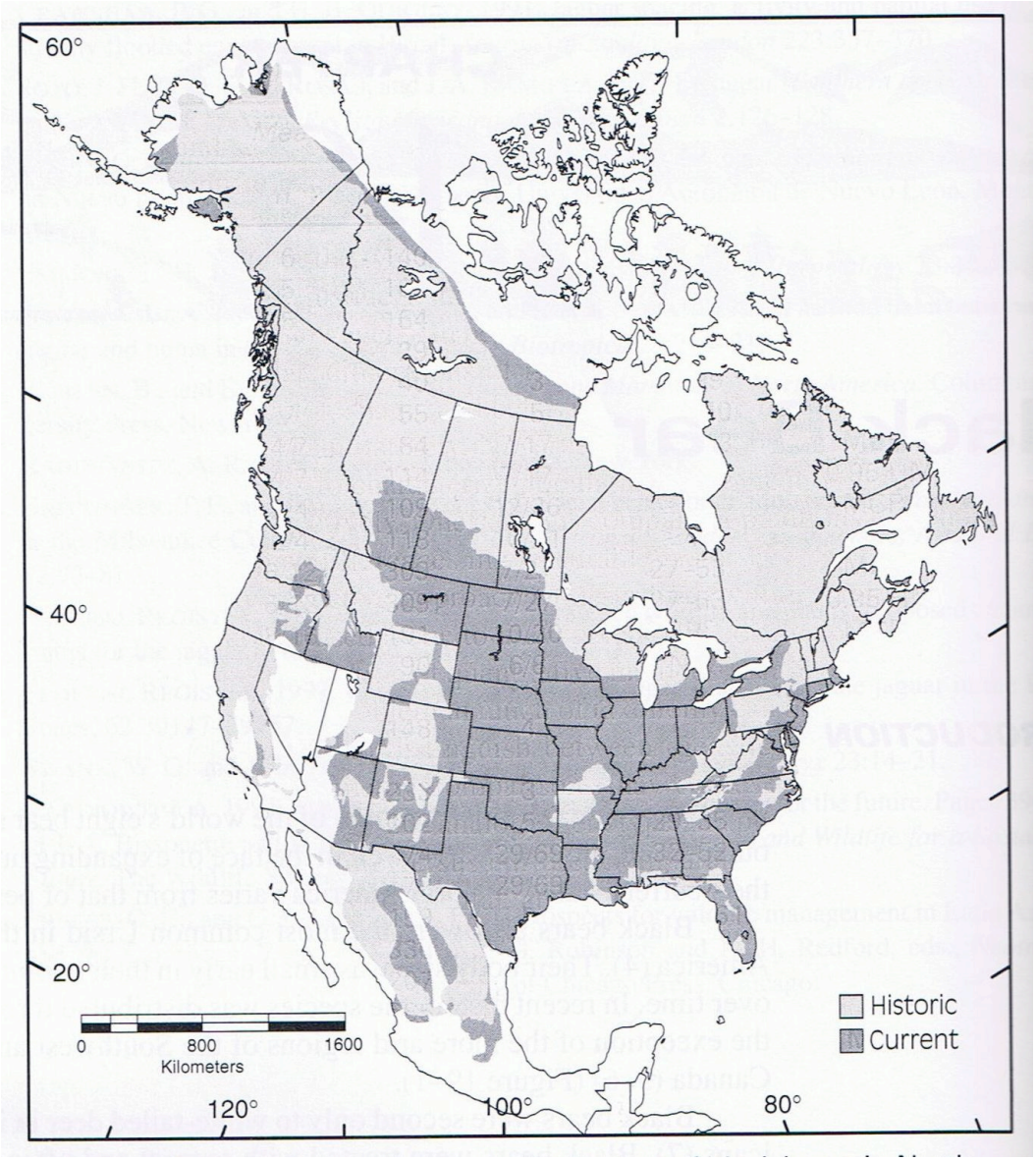

Figure 6. *Ursus americanus* Historical and Current (Modern) Distribution. Although this map is correctly reprinted from Pelton (2000), discussion in text and references to other sources suggest that the current and historic distributions are reversed on this map (Reprinted from Pelton 2000).

Figure 7. Ashland Quadrangle Map Section 21 (Lawson Cave marked with a circled *x*).

Figure 8. Materials Being Bucketed Out of Lawson Cave (circa 1959).

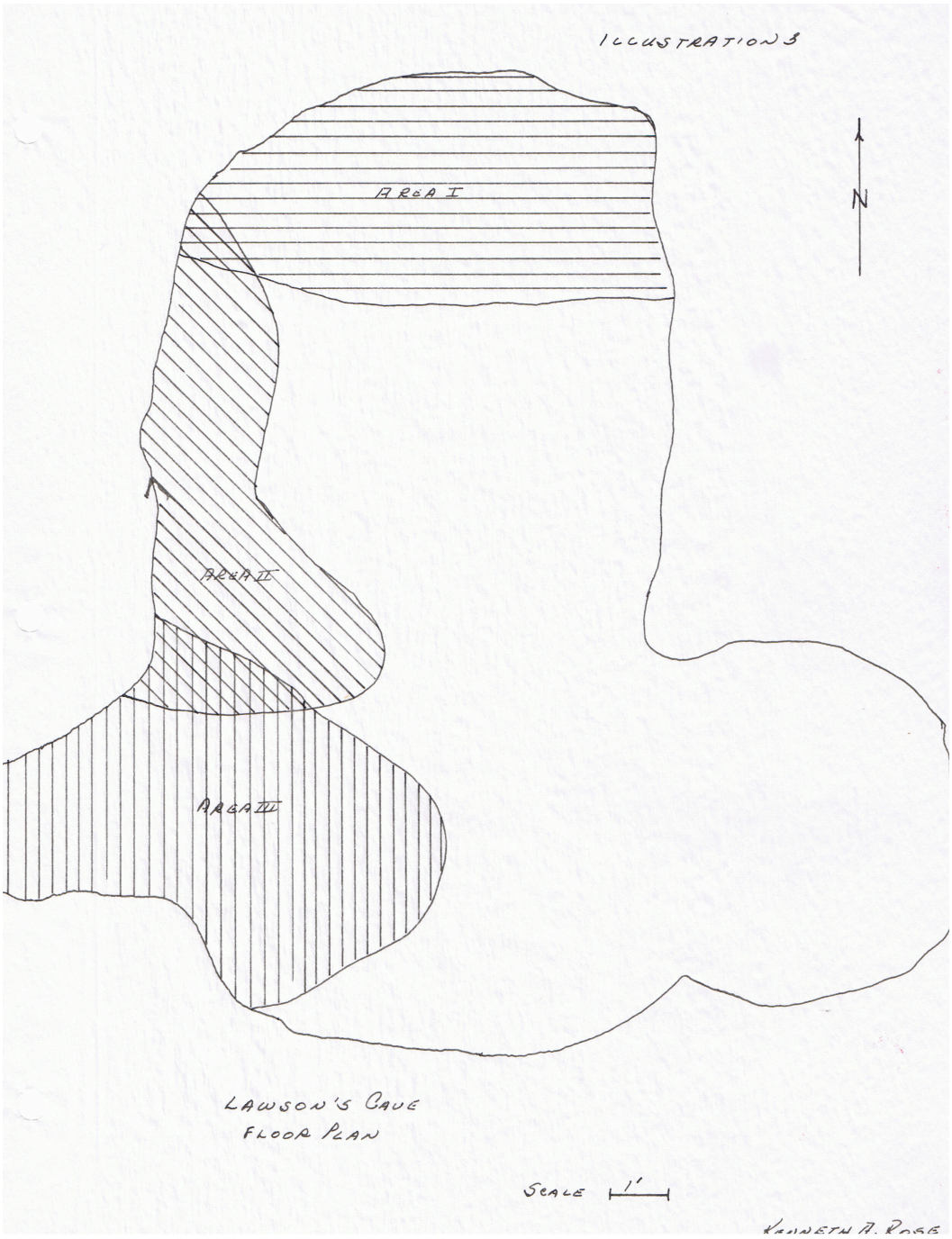

Figure 9. Lawson Cave Floor Plan. Cave entrance is over the Southeast circular portion. Areas I-III signify excavation stages.

Figure 10. Lawson Cave Entrance.

Figure 11. Lawson Cave Entrance (taken from the cave floor).

5’- ACATGAATTCGGAGGGCAACCAGTAGAACACCCCTTTATCATTATCGG

ACAGCTGGCCTCTGTCCTCTACTTCACAATCCTCCTAGTGCTCATGCCC

ATCGCTGGGATCATTGAAAATAACCTCTCAAAATGAAGAGTCTTTGTA

GTATAGTAATTACCTTGGTCTTGTAAGCCAAAAACGGAGAATACCTAC\

CCTCCCCAAGACTCAAGGAAGAAGCAACAGCCCCACTATTAACACCC

AAAGCTAATGTTCTATTTAAACTATTCCCTGGTACATACCATTATTTTA

CCCTGCGTCCTATTCATTTCATATATACC**ACTCTATGTACTGTACCA**
>>>>>>**5F2**>>>>>>>>>> >>>>>>>>>>>>>>**5F**>>>>>>
TCGTAGTATGTTTTTAAATACTTTCC TCTTTTATTTTTTTTCCTCCCCC
>>>>>>>>>>>>>>>>
TATGTACGTCGTGCATTAATGG**CGTGCCCCATGCATATAAG**CATGTA
>>>>>>>>**6F**>>>>>>>>>>>>>>>
CAT A**TTGTGCTTGGTCTTACATGAGG**ACCTACATTTCAAAAGCTTG
<<<<<<<<<<<<<<**5R**<<<<<<<<<<<<<
TTTTGAGTATATGGTCTGTAAGCA**TGTATTTCACTT**<u>**AGTCCGGGAGC**</u>
>>>>>>>>>>>>>7F>>>>>>>>>>>>>>>
<u>**TTGATCAC**</u>**CAGGCCTCG**AGAAACCAGCAACCCTTGCGAGTACGTATA
>>>>><<<<<<<<<<<<**6R**<<<<
CCTCTTCTCGCTCCGGGCCCATGAAGTGTGGGGGTTTCTATGTTGAAAC

TATACCTG**GCATCTGGTTCTTACCTCAGG** – 3’
<<<<<<<<<<<<**7R**<<<<<<<<<<<<<<

Figure 12. Full Sequence of the Control Region of the *Ursus americanus* Mitochondrion (GenBank Accession: AY334364). The selected region is in bold. Forward primer segments are underscored with >>, reverse primer segments with <<. Redesigned primers (5F2 and 6R2) are underlined.

```
HB - CTGCGTCCTATTCATTTCATATATACCACTCTATGTACTGTACCATCGTAGTATGTTTTTAAATACTT
Ua1- ------------------------------------------------------------------------
Ua2- ------------------------------------------------------------------------
Ua4- ------------------------------------------------------------------------
Ua5- ----------------------------C--T------------------------------------

HB - TCCTCTTTTATTTTTTTCCTCCCCCTATGTACGTCGTGCATTAATGGCGTGCCCCATGCATATAAGCA
Ua1- ------------------------------------------------------------------------
Ua2- ------------------------------------------------------------------------
Ua4- ------------------------------------------------------------------------
Ua5- ------------------------------------------------------------------------

HB - TGTACATATTGTGCTTGGTCTTACATGAGGACCTACATTTCAAAAGCTTGTTTTGAGTATATGGTCTG
Ua1- ------------------------------------------------------------------------
Ua2- ------------------------------------------------------------------T--
Ua4- ------------------------------------------------------------------------
Ua5- ------------------------------------------G-----------------------

HB - TAAGCATGTATTTCACTTAGTCCGGGAGCTTGATCACCAGGCCTCGAGAAACCAGCAACCCTTGCGAG
Ua1- ------------------------------------------------------------------------
Ua2- ----------------------------------T----------------------T--------
Ua4- ------------------------------------------------------------------------
Ua5- ------------------------------------------------------------------------

HB - TACGTGTACCTCTTCTCGCTCCGGGCCCATGAAGTGTGGGGGTTTCTATGTTGAAACTATACCTGGCA
Ua1- ------------------------------------------------------------------------
Ua2- -----------T----T-------------------------------------------T------
Ua4- ------------------------------------------------------------------------
Ua5- ------------------------------------------:C------------------------

HB - TCTGGTTCTTA
Ua1- -----------
Ua2- -----------
Ua4- -----------
Ua5- -----------
```

Figure 13. Total Sequence Alignments for Haplotype B (GenBank Accession: AY334364) and All Four Ancient Samples. Haplotype B (HB) represents reference sequence. – indicate similarity to the HB sequence, letters indicate a difference between the sample and the HB sequence, : indicates a deletion. Sequence is aligned beginning at position 267 of the 5' end of the GenBank sequence.

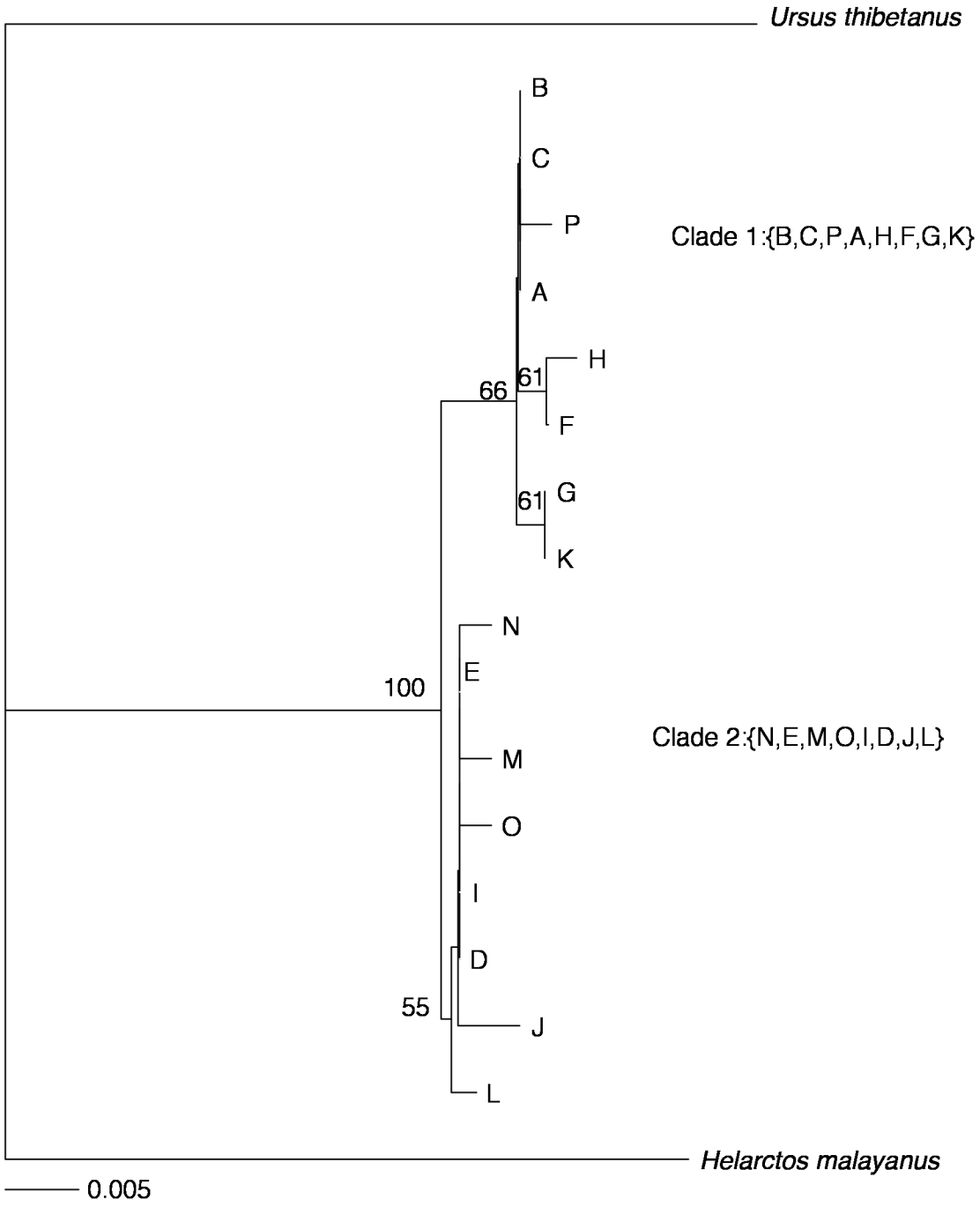

Figure 14. Phylogram for Haplotypes A-P Excluding the Lawson Cave Sequences. Numbers represent bootstrap values. Branches without numbers have <50% support. Outgroups are *Helarctos malayanus* (GenBank Accession: EF196664) and *Ursus thibetanus* (GenBank Accession: NC009971).

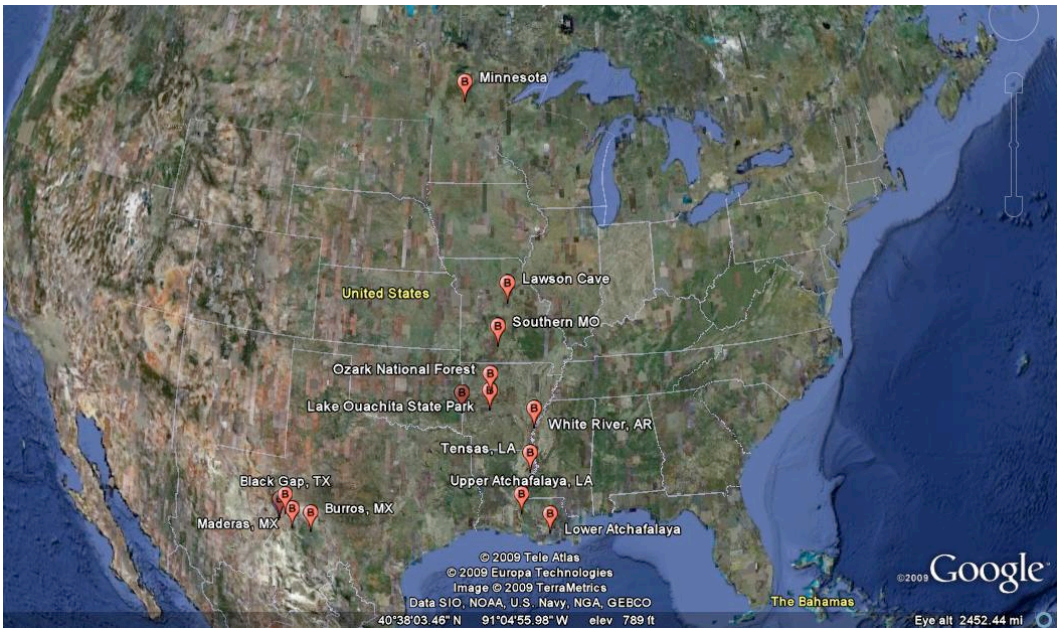

Figure 15. Distribution of Haplotype B Across Central North America. Markers indicate the known occurrence of Haplotype B in that particular locale.

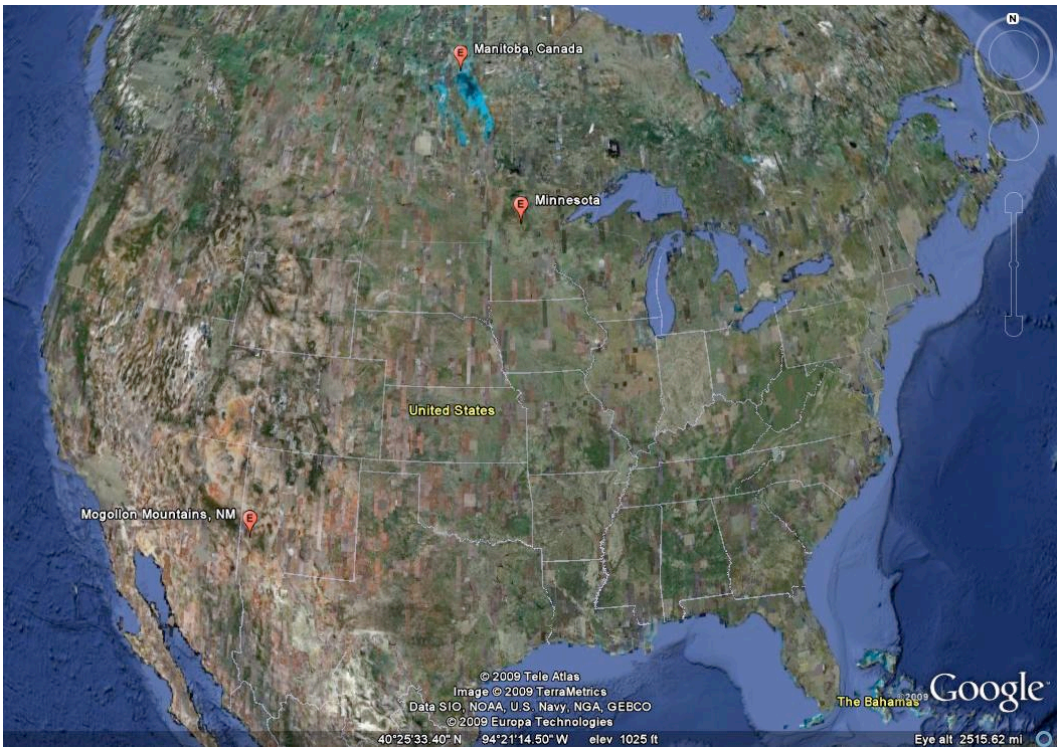

Figure 16. Distribution of Haplotype E Across Central North America. Markers indicate the known occurrence of Haplotype E in that particular locale.

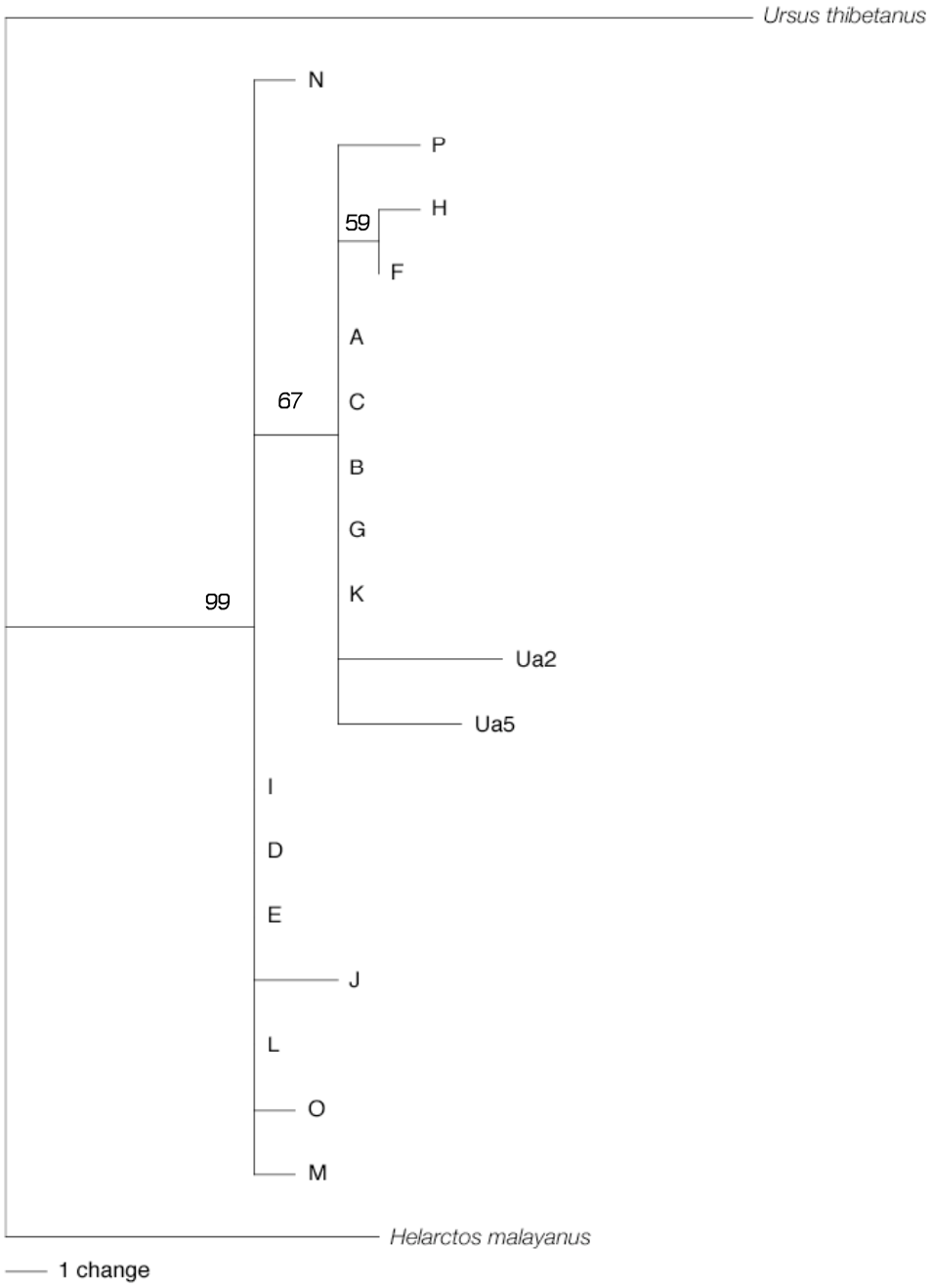

Figure 17. Phylogram for Haplotypes A-P Including the Lawson Cave Sequences. Numbers represent bootstrap values. Branches without numbers have <50% support. Outgroups are *Ursus thibetanus* (GenBank Accession: NC009971) and *Helarctos malayanus* (GenBank Accession: EF196664).

Table 1.1 Primers used to amplify portions of the mitochondrial control region in *Ursus americanus*. See Figure 12 for locations.

Primer	Sequence	Annealing Temp. (ºC)
5F	5'-ACTCTATGTACTGTACCATCGTAGATGTT	58.5
5F2	5'-CCCTGCGTCCTATTCATTTC	53.6
5R	5'-CCTCATGTAAGACCAAGCACAA	53.6
6F	5'-CGTGCCCATGCATATAAG	58.5
6R	5'-CGAGGCCTGGTGATCAAG	58.5
6R2	5'-GTGATCAAGCTCCCGGACTA	55.0
7F	5'-TGTATTTCACTTAGTCCGGGAG	58.5
7R	5'-CCTGAGGTAAGAACCAGATGC	58.5

Table 2.1 Genetic success/failure of each sample as well as their fluorine values and AMS date.

Samples	Fluorine concentration (ppm) (mean±sd)	Ampification (success/failure)	Age (^{14}C yr B.P.) (mean±sd)
Ua2	*390.10 ± 34.00*	*Success*	*229 ± 41*
Ua4	*444.34 ± 16.41*	*Success*	
Ua5	*646.98 ± 11.59*	*Success*	*206 ± 41*
Ua1	*731.69 ± 28.14*	*Success*	
Ua9	780.60 ± 23.36	Failure	
Ua3	898.55 ± 48.56	Failure	
Ua6	906.30 ± 5.90	Failure	
Ua7	959.17 ± 171.08	Failure	
Ua8	1114.14 ± 35.63	Failure	*630 ± 42*
Ua10	1797.61 ± 71.11	Failure	

Table 3.1 Geographic regions used in this study and the haplotypes present (k is the number of haplotypes in each location) (Van den Bussche 2009).

Location	Haplotypes Present	k
Manitoba	D, E, G, I, J, K, M	7
Minnesota	B, D, E, F, L, N	7
White River, AR	B	1
Ozarks, AR	B, E, H	3
Ouachita, AR	B, E, F	3
Ouachita, OK	B, E, F, H	4
Upper Atchafalaya, LA	B	1
Tensas, LA	B, C	2
Lower Atchafalaya, LA	B, C	2
Big Bend, TX	A, B	2
Black Gap, TX	B	1
Trans-Pecos, TX	A, B, C, D	4
Mogollons, NM	D, E	2
Burros, MX	A, B	2
Maderas, MX	A, B	2
Southern MO	B, E, O, P	4
Lawson Cave, MO	B, Ua2 (Q), Ua5 (R)	3

Table 4.1 Haplotypes used in this study and the geographic regions in which they are present (n is the number of locations where each haplotype is found) (Van den Bussche 2009).

Haplotypes	Region/Site	n
A	Big Bend, TX; Trans-Pecos, TX; Burros, MX; Maderas, MX	4
B	Minnesota; White River, AR; Ozarks, AR; Ouachita, AR; Ouachita, OK, Upper Atchafalaya, LA; Tensas, LA; Lower Atchafalaya, LA; Big Bend, TX; Black Gap, TX; Trans-Peco, TX; Burros, NM; Maderas, MX, Southern MO; Lawson Cave, MO	15
C	Tesas, LA; Lower Atchafalaya, LA	2
D	Manitoba; Minnesota; Trans-Pecos, TX	3
E	Ozarks, AR; Ouachita, AR; Mogollons, NM; Manitoba; Minnesota, Southern MO	3
F	Ouchita, AR; Ouachita, OK	2
G	Manitoba	1
H	Ozarks, AR; Ouachita, OK	2
I	Manitoba	1
J	Manitoba	1
K	Manitoba	1
L	Minnesota	1
M	Manitoba	1
N	Minnesota	1
O	Southern MO	1
P	Southern MO	1
Q	Lawson Cave, MO	1
R	Lawson Cave, MO	1

Table 5.1. Polymorphic sites for each of the 13 GenBank haplotypes for central North American black bears as well as the 4 Lawson Cave sequences. The second row is the reference row, - indicate similarity to the reference row, letters indicate a base-pair polymorism, ? indicate missing data, : indicates a deletion.

Sequence Position	36	51	93	261	294	297	314	326	337	345	347	352	353	413
Haplotypes	T	A	T	T	A	C	G	T	C	T	:	:	T	T
A	-	-	-	-	-	-	-	-	-	-	-	T	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	-		:	-
D	C	-	C	-	-	-	-	-	-	-	-	-	-	-
E	C	-	C	-	-	-	-	-	-	:	-	-	-	-
F	-	G	-	-	-	-	A	-	-	-	-	-	-	-
G	-	-	-	C	-	-	-	-	-	-	T	T	-	-
H	-	G	-	-	-	-	A	-	T	-	-	-	-	-
I	C	-	C	-	-	-	-	-	-	-	-	T	-	-
J	C	-	C	-	-	-	-	A	-	:	-	-	-	C
K	-	-	-	C	-	-	-	-	-	-	-	T	-	-
L	C	-	C	C	-	-	-	-	-	-	-	-	-	-
M	C	-	C	-	-	-	-	-	-	-	-	-	-	-
Ua1	?	?	?	?	-	-	-	-	-	-	-	-	-	-
Ua2	?	?	?	?	-	-	-	-	-	-	-	-	-	-
Ua4	?	?	?	?	-	-	-	-	-	-	-	-	-	-
Ua5	?	?	?	?	C	T	-	-	-	-	-	-	-	-

Table 5.2. Polymorphic sites for each of the 13 GenBank haplotypes for central North American black bears as well as the 4 Lawson Cave sequences. The second row is the reference row, - indicate similarity to the reference row, letters indicate a base-pair polymorism, ? indicate missing data, : indicates a deletion.

Sequence Position	415	449	463	467	470	509	532	552	557	583	584	602
Haplotypes	G	A	A	G	C	C	C	C	C	T	T	C
A	-	-	-	-	-	-	-	-	?	?	?	?
B	-	-	-	-	-	-	-	-	?	?	?	?
C	-	-	-	-	-	-	-	-	?	?	?	?
D	-	-	G	A	-	-	-	-	?	?	?	?
E	-	-	G	A	-	-	-	-	?	?	?	?
F	-	-	-	-	-	-	-	-	?	?	?	?
G	-	-	-	-	-	-	-	-	?	?	?	?
H	-	-	-	-	-	-	-	-	?	?	?	?
I	-	-	G	A	-	-	-	-	?	?	?	?
J	-	-	G	A	-	-	-	-	?	?	?	?
K	-	-	-	-	-	-	-	-	?	?	?	?
L	-	-	G	A	-	-	-	-	?	?	?	?
M	A	-	G	A	-	-	-	-	?	?	?	?
Ua1	-	-	-	-	-	-	-	-	-	-	-	-
Ua2	-	-	-	-	T	T	T	T	T	-	-	-
Ua4	-	-	-	-	-	-	-	-	-	-	-	-
Ua5	-	G	-	-	-	-	-	-	-	:	C	T

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