Conservation genetics of grizzly bears
by Frank Lance Craighead

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences
Montana State University

Abstract:
Grizzly bears have some behavioral characteristics that should tend to reduce the amount of genetic variation passed on from generation to generation: females tend to establish home ranges adjacent to their mother and not all males breed. Some males, however, travel widely and breed with several females. In order to examine the genetics of a virtually undisturbed grizzly bear population in the Alaskan Arctic, to determine basic population genetics parameters, and to answer questions of paternity, reproductive success, and genetic population subdivision, I used two DNA 'fingerprinting' techniques. I report data from analyses of multi-locus minisatellite polymorphisms and single-locus microsatellite loci from 152 grizzly bears (including 30 grizzly bear family groups) in the primary study area. I compare these data with smaller samples from 3 other areas. These analyses were made possible by the use of single-locus primers which amplified both of an individual's alleles at 8 loci, and by detailed knowledge of matemal/offspring relationships which allowed the identification of paternal alleles. The alleles examined are shown to be selectively neutral, and distributed in Hardy-Weinberg proportions.

The data demonstrate that each cub in a litter can be sired independently and that one third of all possible litters had multiple sires. Estimates of maximum reproductive success for males indicate that no single male is responsible for more than 11%-13% of total paternity. No more than half of breeding-age males successfully bred. Examination of genotype frequencies, genetic structure and effective population size showed no evidence of genetic structure within any of the populations and no significant difference in heterozygosity between any populations. The data indicate that high levels of heterozygosity (75%) and gene flow throughout grizzly bear range is maintained by the male segment of the population, and they contribute to an understanding of the genetic and demographic basis of male reproductive success which is of vital importance in the maintenance of small, isolated grizzly bear populations.

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A thesis submitted in partial fulfillment
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APPROVAL

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Frank Lance Craighead

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Grizzly bears have some behavioral characteristics that should tend to reduce the amount of genetic variation passed on from generation to generation: females tend to establish home ranges adjacent to their mother and not all males breed. Some males, however, travel widely and breed with several females. In order to examine the genetics of a virtually undisturbed grizzly bear population in the Alaskan Arctic, to determine basic population genetics parameters, and to answer questions of paternity, reproductive success, and genetic population subdivision, I used two DNA 'fingerprinting' techniques. I report data from analyses of multi-locus minisatellite polymorphisms and single-locus microsatellite loci from 152 grizzly bears (including 30 grizzly bear family groups) in the primary study area. I compare these data with smaller samples from 3 other areas. These analyses were made possible by the use of single-locus primers which amplified both of an individual's alleles at 8 loci, and by detailed knowledge of maternal/offspring relationships which allowed the identification of paternal alleles. The alleles examined are shown to be selectively neutral, and distributed in Hardy-Weinberg proportions.

The data demonstrate that each cub in a litter can be sired independently and that one third of all possible litters had multiple sires. Estimates of maximum reproductive success for males indicate that no single male is responsible for more than 11%-13% of total paternity. No more than half of breeding-age males successfully bred. Examination of genotype frequencies, genetic structure and effective population size showed no evidence of genetic structure within any of the populations and no significant difference in heterozygosity between any populations. The data indicate that high levels of heterozygosity (75%) and gene flow throughout grizzly bear range is maintained by the male segment of the population, and they contribute to an understanding of the genetic and demographic basis of male reproductive success which is of vital importance in the maintenance of small, isolated grizzly bear populations.

INTRODUCTION

Around the time of the most recent, Wisconsin, glaciation of the Pleistocene, over 12,000 years before present (ybp), Homo sapiens and Ursus arctos began a long journey together. In uneasy proximity to each other they crossed the Bering Land Bridge from Asia and found great expanses of habitat available. Both species started to populate the New World with their offspring. Homo sapiens has proven to be much better adapted to this, and in the ensuing centuries, due to higher reproductive rates, greater dispersal ability, direct competition for space, widespread modification of habitat, and superior firepower; has reduced Ursus arctos to remnant populations in mountainous habitat over much of its former range. There is more habitat available for man, including that occupied by the bear. The carrying capacity of the environment is much greater for man than for the bear. In fact, man has not yet reached the carrying capacity of his environment in North America; human populations continue to increase, and man continues to compete with the grizzly bear for space. Man converts grizzly bear habitat into human habitat and the bear has been unable, in over 15,000 years, to adapt its behavior enough to live peacefully in human habitat. If the bear is to survive as a species, it will be because man learns to live peacefully, or at least tolerantly, with the bear.

To begin with, the bear was at a disadvantage. Both species competed for some of the same plant and animal foods, and occupied similar habitat. Although, physically the grizzly is much more formidable, early encounters in Beringia with bands of men armed with spears, knives, and clubs, were probably a standoff. Man was able to protect himself from the grizzly, precariously, and the grizzly, presumably, had more attractive prey. The grizzly (and man) also faced competition to some degree from another bear: Arctodus simus, the great short-faced bear. An ancestor of Arctodus had migrated into the New World during the Pliocene and had radiated into both North and South America. When the grizzly arrived it had to compete directly with the great short faced bear for some resources, and was probably even preyed upon. The short-faced bear was in decline at this time and went extinct along with most of its prey species in the great wave of Pleistocene extinctions; perhaps nudged along this path by the grizzly and man.

As the ice sheets receded, human populations expanded rapidly into North America and continued across the Isthmus of Panama into South America. The grizzly expanded its range at a much slower rate, replacing the short-faced bear and eventually inhabiting most of North America from the eastern seaboard to central Mexico. By this time man had thoroughly occupied both continents and a second wave of migration, across the oceans in boats to the New World, had begun. The grizzly had reached its greatest area of dispersal by about the 1500's. From that point on grizzly numbers have been declining, particularly since the 1600's

when European men with firearms arrived in North America and began removing the grizzly, directly and indirectly, from the periphery of its range. By the 1700's man had greatly reduced the grizzly (or brown bear) in western Europe and populations were declining in eastern Europe. There was no longer an uneasy truce: the grizzly was on the run. This man-caused decline in grizzly populations accelerated in the 1800's with greater availability of firearms and poisons and has continued to the present (Cowan 1972, Servheen 1990).

Before the advent of firearms, men and grizzlies were tolerant, respectful, and wary of one another. Indigenous men studied the bear and learned its ways in order to protect themselves, and incorporated this knowledge into rituals in similar ways in all cultures where human populations and grizzly populations were sympatric. Grizzlies were revered, and feared, and were symbolic of important spiritual values (Hallowell 1926, Clark 1966, Rockwell 1991).

Scientific study of the grizzly bear in North America began in the 1920's when Adolph Murie and his brother Olaus began collecting data in Mount McKinley National Park, Alaska. Early data were recorded incidentally to studies of wolves and ungulates, but after the mid-fifties Adolf concentrated on studying grizzlies (Murie, 1981). In 1952 Frank and John Craighead, began an ecological study of the grizzly population in and around Yellowstone National Park (Craighead 1976, 1979; Craighead and Craighead 1963, 1965, 1972; Craighead et. al. 1974, 1976). This study continued until 1969 and

introduced the use of radio telemetry in wildlife studies. Grizzly bear studies have continued in and around Mount McKinley and Yellowstone Parks and similar research, primarily ecological, demographic and behavioral studies, have focused on virtually all extant grizzly populations in North America. A recent compendium of grizzly bear literature, edited in 1987 (Interagency Grizzly Bear Committee 1987a) lists 1,284 entries concerning various aspects of grizzly bear research.

The longest continual study of grizzly bear ecology and demographics to date was begun by Harry Reynolds with the Alaska Department of Fish and Game in 1977, on a study area in the northern foothills of the Brooks Range in northwestern Alaska. Grizzlies have probably inhabited this area continuously since they first arrived over 12,000 years ago.

Since 1980 I have worked on various facets of this study during parts of six field seasons. As with most research there have been many more questions than answers. With the advent of DNA fingerprinting and its application to wildlife studies we saw the possibility of examining genetic components of this well-studied population. Among the questions we hoped to be able to resolve were the genetic effective size of the population, estimates of population genetics parameters, paternity of cubs with known mothers, and the relative reproductive success of all males thus identified.

Since 1988, with the help and advice of Dr. Ernie Vyse, blood and tissue samples have been collected from this population. In

1990 I worked in the field and then began the laboratory phase of the study in Dr. Vyse's laboratory. In 1991 and 1992 both Dr. Vyse and I participated in field work and continued the laboratory work. I visited the U. S. National Fish and Wildlife Forensic Laboratory in Ashland, Oregon in September of 1991 and learned procedures for using chemiluminescent oligonucleotide probes. This technique results in data consisting of band patterns from DNA fragments of large length (2 to 20 kilobases) from variable-number-of-tandem-repeat (VNTR) loci. This is DNA fingerprinting in the classic sense as described by Jeffreys et. al.(1985a,b). This method and its results will be referred to as **fingerprinting** throughout the text.

After three years of laboratory work, using the best available molecular techniques suited to our goals at the time we began, we were still unable to answer some of our questions; but we had established a genetic baseline for grizzly bear populations using multilocus probes. During the summer of 1993 we learned of additional work using microsatellite analysis of black bear DNA by colleagues at the University of Alberta. In September of 1993 I went to Edmonton to work with a technique developed by David Paetkau with Dr. Curt Strobeck using Polymerase Chain Reaction (PCR) amplification of single microsatellite loci.

This technique provided accurate data on both alleles at 8 loci for virtually all of our genetic samples. I finally was able to resolve sufficient variation in my genetic profiles to determine paternity, distinguish individuals, establish pedigrees, and estimate population genetic parameters. This method and its results will be referred to as

microsatellite analysis throughout the text. Additional work by ourselves and others, using these techniques and newer advances, will eventually extend the pedigrees of this population, and fill in the details of the picture that we have begun painting. We are adding another level of inquiry into our knowledge of the grizzly bear.

At the time of this writing, late in the twentieth century, an ecological point of view is central to the life sciences and has even begun to spread to other areas of human activity such as politics and commerce. Man has begun once again to be concerned about the effects of his activities upon other species. In North America in particular, laws have been enacted limiting the harvest of game animals, and when this proved to be insufficient, further legislation has attempted to ensure that species are not driven to extinction through man's activities. Grizzlies were listed as threatened in the conterminous United States in 1975 and became subject to federal protection and management. Conservation of threatened populations are supervised by the Interagency Grizzly Bear Committee (IGBC) based on research conducted by the Interagency Grizzly Bear Study Team (IGBST) as directed by the Grizzly Bear Recovery Plan and the Interagency Grizzly Bear Guidelines (U.S. Fish and Wildlife Service 1982 and 1993, Interagency Grizzly Bear Committee 1987b, Peek et. al. 1987, Servheen 1990). The grizzly is protected by law from becoming extinct and there is once again an uneasy truce. The truce is uneasy now because it may not be possible for man to preserve the grizzly; despite his best efforts. This dissertation, and the research it reports, are a part of the effort to try and preserve Ursus

arctos, and hopefully a host of other species as well, against the continuing momentum of human demographics and its concomitant habitat alteration.

The term Conservation Genetics in the title of this dissertation is used in the sense that genetics data are the subject of this research, and they are analyzed in the context of populations; but the focus is narrowed to those aspects of genetics which may be most useful in terms of the conservation of genetic diversity. As far as I know, the first use of this term was by Wayne et. al. (1991); Conservation genetics of the endangered Isle Royale gray wolf.

The terms brown bear and grizzly bear can be considered to be interchangeable, but I have tried to consistently use grizzly in reference to North American Arctic and interior populations, and brown bear in reference to coastal and Old World populations. The grizzly population we have studied is large, wild, virtually unhunted, and has persisted since the Wisconsin glaciations: it is a viable population. These data are offered as a genetic baseline, describing a viable population, for comparison with other grizzly populations throughout the world.

BACKGROUND

Bear phylogeny and systematics

There are only eight extant species of bears worldwide. Two lines of evidence for bear phylogeny are reviewed: paleontological and molecular. Both sets of data are roughly concordant and in general agreement as to the timing of speciation events.

Paleontological basis of bear phylogeny

The genus Ursus arose in the Old World, probably in Eurasia, in the early Pliocene; probably derived from the Holarctic Miocene genus Ursavus (Kurten and Anderson, 1980). During the early Miocene in Asia, about 22 mybp, the ancestral Ursidae split into two subfamilies, the Ailuropodinae (represented by one extant species the Giant Panda, Ailuropoda melanoleuca) and the Ursinae. Later in the Miocene, the Ursinae radiated into two groups; the Tremarctinae and the Ursinae. The Tremarctinae probably evolved by vicariance as a result of crossing the Bering Land bridge into the New World and becoming isolated, probably about 15 mybp. Two genera, Tremarctos and Arctodus evolved in the New World, eventually becoming extinct in North America. Tremarctos survives in South America as the spectacled bear, Tremarctos ornatus.

During the Pliocene, about 3.5 mybp, the first ancestral Ursine bear migrated into the New World. The fossil record indicates that it evolved into the American black bear, Ursus americanus, and was a separate species by at least 1.5 to 2.5 mybp (Kurten 1964, 1968, 1976. Kurten and Anderson 1980), establishing itself south of the ice sheets during the Pleistocene glaciations. It shared this area with three species of Tremarctinae; Tremarctos floridanus, Arctodus pristinus, and Arctodus simus. U. americanus is the most commonly found Pleistocene bear species among the fossils from this period. A. simus, the giant short-faced bear was the most wide-ranging bear species during the Pleistocene in North America, and was present in Alaska and the Yukon during the late Pleistocene. The northern forms were very large and it was the most powerful predator of the Pleistocene fauna of North America (Kurten and Anderson, 1980).

An ancestor of the black bear, which remained in the Old World radiated into southern Asia, also during the Pliocene, and evolved into two or more lineages that are represented by three extant species: the Malaysian sun bear, Ursus malayanus (or Helarctos), which probably became isolated on the Malay Peninsula; the Asiatic or Indian sloth bear, Ursus ursinus (or Melursus), which probably became isolated on the Indian subcontinent; and the Asiatic black bear, Ursus thibetanus (or Selenarctos), which may have become isolated on the Tibetan plateau or elsewhere in the Himalayas.

The grizzly bear, Ursus arctos, evolved in the Old World from the ancestral Ursus etruscus during the middle Pleistocene about 1.6

mybp. U. arctos radiated again about 300,000 ybp to form the polar bear, Ursus maritimus, probably in northern Asia (Kurten 1964, 1968, 1976. Kurten and Anderson 1980). The grizzly crossed the Bering Land Bridge during the Wisconsin glaciation, about 13,000 ybp. The earliest fossil dates in North America are from 12,950 +- 550 ybp from Welsh Cave. It was found only north of the continental ice sheets during the Pleistocene. It coexisted with A. simus during the Rancholabrean in the Alaska-Yukon refugium, and finally migrated below the ice sheets only during the last phase of glaciation as the ice sheets receded and the Mackenzie Corridor opened up. The only known association of these 2 species south of Alaska is from Little Box Elder Cave near Douglas Wyoming (Kurten and Anderson 1980). The grizzly was probably in competition with the giant short-faced bear throughout its range and eventually replaced it: the latest fossil record for A. simus is 12,650 +- 350 ybp from Lubbock Lake. As the grizzly expanded its range south of the ice sheets it competed with the black bear and restricted the black bear's range. By the time of the earliest historical records, the grizzly had expanded as far south as Mexico.

Genetic evidence of bear phylogeny

Phylogenetic studies of bear genetics began with the work of Fred Allendorf and his colleagues at the Institute of Ecology and Genetics, University of Aarhus, Denmark (Allendorf, et. al. 1979). The original work focused on electrophoretically detectable protein

variation (or the lack of it) in polar bears. Thirteen loci were examined in 52 individual polar bears with no allelic variation observed. Subsequent work has been continued in Allendorf's laboratory at the University of Montana comparing allelic differences using protein electrophoresis among the three species of North American bears.

Manlove and colleagues examined biochemical variation in the black bear and presented results at the 4th International Conference on Bear Research and Management (Manlove et. al. 1980). Further protein comparisons between polar bear populations were made by Larsen et. al. (1983).

Stephen J. O'Brien's group at the National Institute of Health laboratories also took this approach. The first results were reported in 1987 with the publication of molecular genetic estimates among the Ursidae using protein electrophoresis (Goldman et. al. 1987, 1989). Allelic differences were examined among 289 fibroblast proteins and 44 isozyme loci. These results were followed by Nash and O'Brien's (1987) karyotype comparisons among the Ursidae and other carnivores which indicated that three major chromosomal reorganization events had occurred during the evolutionary history of modern ursids. The first was a multichromosomal fissioning which increased the chromosome number from 2n=44 in the primitive carnivore karyotype (Dutrillaux and Couturier, 1983) to 2n=74 in the ancestral Ursidae. The second was a comprehensive chromosome fusion in the lineage that led to the Ailuropodinae subfamily and resulted in 2n=44 but with chromosomes distinct

from the ancestral form. The third event was another, independent centromeric fusion in the lineage which led to the Tremarctinae subfamily and resulted in 2n=52. The karyotype of 2n=74 remains in all six species of the Ursinae subfamily (Nash and O'Brien, 1987).

The chronology of these genetic events agrees with the paleontological data if the rates of protein divergence in bears are assumed to be equivalent to that found in primates, a more intensively studied group. Under this assumption, between 22.4 and 32.3 mybp an ancestor of the Procyonids (raccoon) and Ursids split into these two lineages. Subsequently an ancestor of the Ailuropodinae split from the Ursid line about 18 to 22 mybp. An ancestor of the Tremarctinae split from the Ursid line about 10.5 to 15.0 mybp. The six extant species of ursine bears have diverged from a common ancestor within the past 4 to 8 million years but this radiation was not resolved by protein electrophoresis (Goldman et. al. 1989).

The use of mitochondrial DNA (mtDNA) to examine Ursid phylogenetics was undertaken by Gerald Shields and Thomas Kocher (1991) who compared restriction fragments of whole mtDNA as well sequence descriptions of cytochrome b genes and mtDNA control regions among American black bears, brown bears and polar bears. Their work involved the first use of the Polymerase Chain Reaction (PCR) and nucleotide sequence analysis to compare bear species. Their results agreed with the fossil evidence, protein electrophoresis results, and the fact that brown and polar bears produce fertile F1 hybrids in captivity (Kowalska 1965); i.e. that brown and polar bears

are sister taxa. If divergence of mtDNA is assumed to occur at a similar rate to primates, then the black bear lineage split from the polar bear-brown bear line about 3.8 million years ago. Polar bear and brown bear speciation occurred within the last million years (Shields and Kocher 1991).

Matthew Cronin and colleagues compared mtDNA haplotypes among the three species of North American bears. Brown bears and polar bears were found to share similar mitochondrial DNA (0.23 base substitutions per nucleotide) which is quite divergent (0.78 base substitutions per nucleotide) from that of black bears. Brown and polar bears were demonstrated to be paraphyletic with regard to mtDNA. Although black bears and polar bears have relatively low levels of protein variation, there is considerable mtDNA variation in all three bears compared with other mammals. Currently designated grizzly subspecies are not characterized by different mtDNA haplotypes, and one haplotype found in Southeastern Alaska grizzly bears is more closely similar to those of polar bears than to other grizzlies. Because of the close relationships of the three species and the large amount of variation, Cronin et. al. (1991a) feel that assuming a sequence divergence rate similar to primates is unwarranted.

Taberlet and Bouvet (1992) developed a technique to amplify mtDNA sequence and were able to apply it to a single hair from a Pyrenean brown bear (Taberlet and Bouvet 1992) They then examined sequence variation in European brown bear mtDNA from 60 bears. Sequence divergence percentages indicated that two

European lineages diverged about 850,000 ybp during the first ice age. Catherine Hanni was able to amplify a 140 base-pair fragment of mtDNA from cave bear bones which indicated that this species appeared at about the same time (Dorozynksi 1994). Both these results are somewhat tentative because of the small sample sizes involved.

Interspecies studies of bear genetics

Allendorf and Knudsen have examined isozyme variation in grizzly bears and polar bears. Varying levels of polymorphism were found in different populations. Least variation was found in grizzly bears from Kodiak Island (Knudsen 1992)

Zimmerman (1989) examined mtDNA differences among 55 black bears using 10 restriction enzymes and comparing the resulting restriction fragment length polymorphisms (RFLPs). Minor differences were found between three subspecies, Ursus americanus americanus, U. americanus floridanus; and the more restricted Louisiana populations of U. americanus luteoulus in the Atchafalaya basin.

Cronin et. al. (1991a) found 6 mtDNA haplotypes in black bears, 5 in brown bears, and 4 in polar bears from sample locations in North America. Black bears (n=40) examined were from Alaska, New Hampshire, Oregon, and Montana. Brown bears (n=60) were from Alaska and Montana, and polar bears (n=40) were from Alaska and the Northwest Territories. Two genetically distinct groups of

black bear haplotypes were found, and some types were found in only one location.

The 2 morphological forms of brown bears, interior grizzly and coastal brown bears, do not cluster as distinct mtDNA lineages, and as mentioned above, one brown bear haplotype is more closely related to polar bears than to other brown bears. One haplotype was found only in Montana, one only in Southcentral Alaska, one only in northwestern Alaska (Seward peninsula), and one only in islands of southeastern Alaska. One haplotype was found in all 26 samples from Kodiak Island, but also from some samples from the nearby Alaska mainland.

Two polar bear haplotypes were found in all 3 sample locations while one haplotype was found only in Alaska and one was found only on Ellesmere Island, Canada. These geographic distributions are considered preliminary in all three species because of small sample sizes (Cronin et. al. 1991a).

Currently, Sandy Talbot and Gerry Shields are conducting sequence analysis of grizzly bear Mitochondrial DNA looking at the control region of the D loop and the cytochrome B gene. At the time of this writing Talbot is determining mtDNA sequence for most of the 30 females for which we have microsatellite pedigree data (Shields and Talbot, pers. comm.).

Related wildlife genetic studies

For the purposes of our study we needed a molecular technique that would resolve finer differences among individuals

than either protein electrophoresis or mtDNA analysis. DNA restriction fragment length polymorphisms (RFLPs) have been invaluable genetic markers for use in linkage analysis (White et. al. 1985), medical diagnosis, and cancer research among other applications (Cronin et. al. 1991b), but their variability was generally too low for analysis of pedigrees until the fortuitous discovery of variable number of tandem repeat (VNTR) loci or 'minisatellite' DNA (Wyman and White, 1980).

These hypervariable regions were found to consist of a DNA sequence found in multiple copies; allelic differences result from variation in the number of repeats, probably due to unequal recombination events. The technique was first developed by Jeffreys lab (Jeffreys et. al. 1985a) to describe unique genetic profiles of individuals using human DNA. Jeffreys and co-workers described a human minisatellite comprised of repeats of a 33-base pair sequence (Weller et. al. 1984) which was used to prepare probes that could detect polymorphisms in human DNA digested with HinfI and HaeIII. A 10-15-base pair core sequence may act as a recombination signal (Jeffreys et. al. 1985a). Most minisatellite repeat units used for fingerprinting are 9 to 60 base pairs in length (Weber and May 1989).

Three probes (33.5,33.6, and 33.15) were cloned and characterized which consisted of tandem repeats of various versions of the core sequence. The fragments detected were found to be inherited in a Mendelian fashion (Jeffreys et. al. 1985a). The pattern of fragments which hybridized to a given probe was designated a

DNA 'fingerprint'. 'DNA fingerprinting' can thus be defined as the use of detectable DNA probes which hybridize to these hypervariable tandem repeat, or VNTR, segments (Wyman and White 1980) of genomic DNA.

Fingerprints derived from these multi-locus probes were shown to be individual specific, and could be used to determine first-order genetic relationships: paternity, maternity, and sib-ship (Jeffreys et. al. 1985b). However, an assessment of paternity requires a detailed knowledge of the study population and DNA samples from both parents and offspring (Wetton et. al. 1987). Fingerprinting data is inaccurate for more distant relationships (Lynch 1988)

Subsequently, Jeffreys and co-workers were able to clone DNA fragments from DNA fingerprints to provide locus-specific probes (Wong et. al. 1986). Human probes have been shown to hybridize to animal DNA and the multi-locus VNTR probes have been used to develop pedigrees of dogs and cats (Jeffreys and Morton 1987), and mice (Jeffreys et. al. 1987). Wetton et. al. (1987) and Burke and Bruford (1987) used Jeffreys probe 33.6 to demonstrate multiple paternity in house sparrows. Jeffreys' probe 33.15 also hybridizes readily with bird DNA.

Vassart et. al. (1987) found that a 280bp tandem repeat in M13 phage DNA would hybridize to hypervariable loci in humans, cows and dogs, and probably salmon. Westneat et. al. (1988) then developed improved conditions for hybridization of M13.

A tandem repeat was purified from merlin DNA and cloned into M13mp8 and pUC8 to develop a probe (pMR-1) for falcon species (Longmire et. al. 1988). This probe revealed highly polymorphic fragment patterns in the family Falconidae but not in the closely related Accipitridae. DNA fingerprints of peregrine falcons were used to differentiate between Greenland and Argentina populations. Longmire et. al. used the M13 probe to develop fingerprints of falcons and to isolate clones in a human chromosome-16-specific library inserted in Charon 40. A 4.5kb fragment of that clone, PV47-2, was subcloned into pUC8 and propogated in JM101 and was found to detect additional bands not detected by the phage repeat (Longmire et. al. 1990).

Quinn et. al. (1987) used sequence probes isolated from a snow goose genomic library to reveal paternity of nestlings. Four probes; M13, Jeffreys 33.15, the human alpha-globin hypervariable region (HVR), and a Drosophila Per probe, were used for animal identification, paternity testing, and linkage analysis in horses, dogs, pigs, chicken, and fish (Georges et. al. 1988).

Multi-locus VNTR probes have been used to determine paternity, and the development of pedigrees for wild populations has been demonstrated with old world monkeys (Weiss et. al. 1988). They have also been used to measure realized reproductive success. A mouse major histocompatibility complex cDNA probe was used in conjunction with two minisatellite probes (Jeffreys' 33.15 and the M2.5 repeat from the mouse Per gene) to determine paternity and estimate reproductive success in red-winged blackbirds (Gibbs et. al.

1990). Similar paternity and pedigree analysis techniques have been used to analyze kinship in prides of Serenghetti lions where the history of each lion pride was known (Packer et. al. 1991a,b).

Jeffreys' probes detect a wide range of variation in different taxa. The mean number of bands detected per probe includes 29.5 in humans using 33.15 or 33.6 (Jeffreys et. al. 1985a,b), 30 in Old World monkeys using 33.15 (Weiss et. al. 1988), 19 in dogs using 33.15 and 16 using 33.6, 13 in cats using 33.15 and 8 using 33.6 (Jeffreys and Morton 1987, Hill 1987), 15 in sparrows using 33.15 and 6 using 33.6 (Hill 1987, Burke and Bruford 1987), 23.7, 17.7, and 18.6 in different species of swan using 33.6 (Meng et. al. 1989), 10 in naked mole rats using 33.15 and 7 using 33.6 (Faulkes et. al. 1990, Reeve et. al. 1990). Bears exhibit less variation using these probes than most other species with a mean of 7.7 to 12.8 in black bears (Fain 1991) and similar amounts in grizzly and polar bears (Fain, pers. comm.).

The techniques of DNA fingerprinting with genomic DNA are also well suited to the analysis of inter-population genetic variation (Lynch 1990, 1991). Jeffreys' 33.6 probe was used to estimate genetic variability and reconstruct evolutionary relationships in small, isolated populations of the California Channel Island Fox (Gilbert et. al. 1990).

A combination of techniques; protein electrophoresis, mtDNA restriction site analysis, and analysis of hypervariable minisatellite DNA using Jeffreys' probe were used by Robert Wayne's group at UCLA to examine the genetics of wolves, particularly the wolves of

Isle Royale in Michigan. The mtDNA analysis found a genotype on Isle Royale that is very rare on the mainland, suggesting that the island population was founded by a single female. The DNA fingerprinting analysis indicated that all the wolves on the island were related and somewhat inbred, being as closely related as captive siblings (Wayne et. al. 1991).

Mitochondrial DNA comparisons among wolf and coyote populations in states and provinces near Isle Royale found an introgression of coyote mtDNA into wolf populations. Apparently female coyotes occasionally have bred with wolves to produce hybrid offspring (Lehman et. al. 1991). Further work with wolf genetics at UCLA used fingerprint similarity (bandsharing) as an index of relatedness among unknown individuals. Wolves were considered unrelated if they had different mtDNA genotypes (Lehman et. al 1992). Individuals (n=104) considered to be related at the level of parent-offspring had similarity values, S, of between 0.700 and 0.889 (mean = 0.785, SE = 0.056).

The fingerprinting studies reported above used genomic DNA fragments cloned into appropriate vectors and then labeled using radioisotopes; primarily Phosphorus-32. DNA fingerprinting results were further improved with the use of DNA synthesis techniques to create shorter-length oligonucletides that could then be labeled with radioisotopes (Zeff and Gellebter 1987). Subsequent advances in the development of non-radioactive, chemiluminescent labeling techniques (Edman et. al. 1988, Zischler et. al. 1989, Nurnberg et. al. 1989) resulted in non-hazardous multi-locus VNTR probes labeled

with chemiluminescence-producing enzymes, primarily alkaline phosphatase. This is the approach I first used to analyze my DNA samples.

The Polymerase Chain Reaction (PCR) was discovered in 1985 and became widely applicable to genetic studies with the use of thermally stable Taq (Thermus aquatilis) DNA polymerase (Saiki et. al. 1985, 1988). Improvements in this technique have made it possible to amplify a target sequence of DNA (up to several hundred base pairs) one million fold (White et. al. 1989). The PCR requires paired primer sequences which anneal to complimentary target DNA sequences after the target DNA has been denatured at 94^0 C. Annealing occurs at about 50^0 C., followed by elongation from the 3' end of the primers, catalyzed by Taq polymerase, at 72^0 C. These 3 successive temperature stages are repeated for 25 to 45 cycles and the amount of PCR product can theoretically be doubled during each cycle (Gyllensten 1989).

Random 10-mer oligonucleotide primers have been used with Taq polymerase to detect polymorphisms in genomic DNA of humans, plants, and bacteria (Williams et. al. 1990). The sequences amplified differ in amount of variation and must be screened carefully in order to find useful primers. DNA microsatellites, or relatively short lengths (<100 bp) of tandemly repeated sequences (1-6 bp long) have been characterized and found to be highly polymorphic in length among human individuals (Weber and May 1989). Most work has been done with families of dinucleotide repeats of the form $(CA)_n$ $(GT)_n$ (Beckman and Weber 1992).