Genetic relationships between white-tailed deer, mule deer and other large mammals inferred from mitochondrial DNA analysis
by Matthew Anthony Cronin

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

Abstract:
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GENETIC RELATIONSHIPS BETWEEN WHITE-TAILED DEER, MULE DEER AND OTHER LARGE MAMMALS INFERRED FROM MITOCHONDRIAL DNA ANALYSIS

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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

Restriction enzyme analyses of mitochondrial DNA (mtDNA) of six artiodactyl species and two bear species were employed to estimate genetic divergence between groups. Estimates of base substitutions per nucleotide were 0.000-0.008 for intra-species, 0.058-0.085 for intra-family, inter-species and 0.113-0.198 for inter-family comparisons. From these, estimates of divergence time between taxa were made which were generally consistent with estimates from the fossil record for species which diverged less than 5 million years ago. My estimates for species with older divergence times are probably underestimates.

Comparisons of white-tailed deer and mule deer in Montana revealed species specificity of serum albumin and mtDNA and a probable low level of inter-species hybridization. Despite the species specificity of mtDNA, genetic divergence estimates between the species in Montana are of the same magnitude as intra-species comparisons of other groups suggesting introgression of white-tailed deer mtDNA into mule deer populations in the past.

INTRODUCTION

Before the development of molecular genetic techniques in the 1950's the assessment of variability within and between natural populations relied mainly on the description and measurement of morphological characters. Morphological traits are usually reliable for indentifcation of species and are traditionally used at all levels of taxonomic classification. For some animals, notably Drosophila spp., rodents and domestic livestock, controlled breeding studies have allowed estimation of the heritabilities of various morphological traits such as coat color or body size (Falconer, 1964; Lewontin, 1974).

To accurately assess the genetic differences within or between populations knowledge of allele frequencies or nucleotide sequences must be available. For some morphological traits, such as Mendel's pea flower colors, single allelic differences correspond to recognizeable phenotypic differences. However, much of observable phenotypes is under the influence of polygenes so simple allelic determinations are not possible. Pleiotrophic effects of single genes may further complicate analysis. Also, in natural populations, the effect of environment on phenotype may be significant and indistinguishable from genetic effects. For example Mayr (1970) notes that British red deer (Cervus elaphus scoticus) transplanted to New Zealand acquired the phenotype of a different race within one or two generations. This general lack of a 1:1 correspondence between

genotype and phenotype prevents enumeration of genotypes and allele frequencies for most natural populations when only morphological traits are studied.

For many extant and fossil large mammals, species and subspecies designations have been based on variations in traits such as size, coloration or horn and antler morphology. For example thirty subspecies of white-tailed deer (Odocoileus virginianus) (Baker, 1984) and eight subspecies of mule deer (O. hemionus) (Wallmo, 1981) are recognized. Knowledge of intra-species morphological variation is useful for speculation on the local adaptiveness of traits or gene flow between areas but usually a quantitative assessment of environmental and genetic contributions to observed variation is not possible.

The use of molecular genetic techniques allows quantitative analysis of actual genetic differences between organisms, although not necessarily assessment of the adaptive value of the variation observed. Morphology, physiology and ecology must also be studied to gain insight into adaptive differences between organisms.

Protein electrophoresis can detect differences in protein size or charge caused by differences in amino acid sequence. Since amino acid sequence is determined by nucleotide sequence, these protein variations can be interpreted as allelic differences at specific loci. Since its first application to population genetics (Harris, 1966; Lewontin and Hubby, 1966) protein electrophoresis has greatly increased our knowledge of genetic variation in natural populations. Examples include identification of population subdivision in white-

tailed deer (Smith et al., 1984) and moose (Alces alces) (Chesser et al., 1982), and genetic differences between subspecies of red deer (Cervus elaphus) (Gyllensten et al., 1983) and sunfish (Lepomis macrochirus) (Avise et al., 1984). Electrophoresis has also been used to identify species-specific forms of various proteins in bears (Wolfe, 1983) and cervids (McClymont et al., 1982), and to estimate the genetic distance between several artiodactyl species (Baccus et al., 1983).

Recently the analysis of nucleic acids has been applied to the study of population genetics and taxonomy. For example, Sibley and Ahlquist (1983, 1984), using DNA-DNA hybridization, have revised the taxonomy of birds and primates, estimating relatedness of groups from similarity of chromosomal DNA sequence.

In the last ten years variations in mitochondrial DNA (mtDNA) have been studied to assess differences between individuals, populations and species. Mammalian mtDNA is a supercoiled circular molecule about 16-17 kilobases (kb) in length. Because of its relatively small size, mtDNA is easily isolated without breakage and the entire molecule can be subjected to analyses. Mammalian mtDNA is structually an extremely conservative molecule compared to nuclear DNA which has long non-coding or duplicative sequences. Almost all of the mammalian mtDNA molecule consists of functional coding sequences (Anderson et al., 1981).

There is substantial evidence that mtDNA is homogeneous within individuals and maternally inherited without meiotic recombination via oocyte cytoplasm (Avise et al., 1979a, 1979b). Possible exceptions to

these tenets have been reported. Coote et al. (1979) found slight differences in mtDNA from the liver and brain of the same ox (Bos taurus). Hauswirth and Laipis (1982) found a variant mtDNA genotype independently arising five times in one maternal lineage of cattle. These authors suggest that the two genotypes in one maternal lineage result from intra-individual heterogeneity possibly due to paternal inheritance, maternal nuclear gene effects on mtDNA or simply segregation of multiple genotypes within an oocyte. Harrison et al. (1985) noted heterogeneity in the size of mtDNA molecules in individual crickets (Gryllus). In mammalian studies, however, intra-individual homogeneity and maternal inheritance seem to be the rule (Lansman et al., 1981).

Mitochondrial DNA from humans (Anderson et al., 1981), cattle (Anderson et al., 1982) and mice (Mus) (Bibb et al., 1981) has been completely sequenced, allowing comparisons of entire mitochondrial genomes. Sequencing is time consuming and expensive, however, and not necessary for routine population genetic analysis. Population studies involving mtDNA usually involve the isolation of mtDNA followed by treatment with restriction endonucleases. These enzymes recognize a specific sequence of 4, 5, or 6 nucleotides and cleave the molecule each time the sequence is encountered. The resulting DNA fragments vary in number and size depending on the number and location of cleavage sites. The DNA fragments are separated on the basis of size by electrophoresis and visualized by staining or autoradiography. The size of the DNA fragments can be estimated by comparisons with standards of known size and patterns of different organisms compared.

A complete review of the technique is given by Lansman et al. (1981).

The type of analysis of digestion patterns recommended for population surveys (Upholt, 1977; Nei and Li, 1979; Lansman et al., 1981) involves, for each different enzyme digestion, identification of fragments common to the individuals compared. The common fragments are assumed to be homologous (generated by cuts at cleavage sites shared by descent). The proportion of shared fragments is calculated and from this the number of base substitutions per nucleotide is estimated as an indication of genetic distance (Upholt, 1977). Brown et al. (1979) and Ferris et al. (1983a, 1983b) estimated the rate of nucleotide substitution in mtDNA to be 2-4% per million years. Using this rate, divergence time between two mitochondrial genomes can be estimated.

Analyses of mtDNA in populations of mice (Peromyscus maniculatus, P. polionotus) and pocket gophers (Geomys pinetis) (Avise et al., 1979a, 1979b) have revealed low levels of mtDNA divergence in local populations and increasing levels of divergence in geographically separated populations and between species. Evidence of introgression of mtDNA from one species or subspecies into another has been found in mice (Mus) (Ferris et al., 1983a), deer (Odocoileus) (Carr, personal communication), sunfish (Lepomis) (Avise et al., 1984), frogs (Rana) (Spolsky and Uzzell, 1986) and Drosophila (Powell, 1983). Wright et al. (1983) used mtDNA analysis to infer that the lizard, Cnemidophorus gularis, was the maternal parental species involved in the creation of C. laredoensis by hybridization with C. sexlineatus.

Broader taxonomic surveys using mtDNA include analyses of genetic

divergences of the mtDNA of human and non-human primates (Brown et al., 1979), sheep (Ovis) and goats (Capra) (Upholt and Dawid, 1977), species of rats (Rattus) (Brown and Simpson, 1981), mice (Mus) (Ferris et al., 1983b) and several breeds of pigs (Sus) (Watanabe et al., 1985). These studies have shown that analysis of mtDNA with restriction endonucleases can be used to estimate the amount of nucleotide sequence divergence between individuals, populations and species.

The fossil record, morphology and protein phenotypes give indications of genetic distance between different North American artiodactyls and ursids. The families Cervidae, Bovidae and Antilocapridae are believed to have split in the Miocene 20-30 million years before the present (mybp) (Romer, 1966). The morphologically distinct cervid subfamilies Cervinae and Odocoilinae may also have diverged in the Miocene (Osborn, 1910; Scott, 1937; Geist, 1981). Many extant species in the cervid, bovid and ursid families are thought to have originated in the Pliocene or Pleistocene (Kurtén, 1968; Kurtén and Anderson, 1980). Genetic distance estimates for artiodactyls from protein electrophoresis generally agree with relative divergence as indicated by morphology and the fossil record (Baccus et al., 1983).

Mule deer and white-tailed deer are sympatric in parts of western North America. Each species has distinctive morphology (Wishart, 1980), behavior (Geist, 1981), habitat preferences (Mackie, 1981) and serum albumin (McClymont et al., 1982). Hybridization between natural populations of these two deer species has been reported in Alberta

(Wishart, 1980) and other areas (Kramer, 1973). Wishart (personal communication) noted morphological characters, particularly metatarsal gland length, as intermediate to either parental type in hybrids from controlled breeding experiments. McClymont et al. (1982) using polyacrylamide gel electrophoresis, identified species-specific serum albumin patterns for the two deer species. Suspected hybrids with intermediate morphologies displayed the heterozygote pattern of two albumin bands, one characteristic of each species. Kramer (1973) felt that hybridization in nature would be minimized by behavioral mechanisms. Geist (personal communication) has noted that the courtship behaviors of the two species are very different and feels that only male whitetail x female mule deer crosses are likely to occur. Hybrids from inter-specific crosses in captivity are fertile (Kramer, 1973; Wallmo, 1981), but Wishart (personal communication) reported infertility, low sperm motility and deformed sperm in F-1 hybrid males while F-1 hybrid females are fertile. To our knowledge there is no documentation of hybrids breeding in the wild.

White-tailed deer and mule deer are found throughout Montana and are often considered as habitat separated. Overlap of ranges does occur and it is not uncommon to see both species together. The species can be considered to be sympatric or parapatric over most of Montana and interbreeding is not necessarily prevented by spatial separation (Mackie, personal communcation).

This study was conducted to test the hypothesis that mule deer and white-tailed deer are genetically distinct, reproductively isolated species and to characterize inter-specific gene flow if it

occurs. In addition, I applied restriction endonuclease analysis of mtDNA to several cervid (elk, Cervus elaphus, white-tailed deer, mule deer), bovid (bison, Bison bison, cattle), antilocaprid (pronghorn, Antilocapra americana) and ursid (black bear, Ursus americanus, grizzly bear, U. arctos) species to estimate the degree of sequence divergence and time of divergence of the taxa.

MATERIALS AND METHODS

Sample collection

Tissues (50-100 grams (g) of liver, kidney, brain, or skeletal muscle) were collected from animals killed by hunters or in culling operations during 1984-85 and frozen at -20C. For mule deer and white-tailed deer, species identification was made by biologists or hunters. Table 1 lists the species and locations sampled.

Albumin electrophoresis

Mule deer, black-tailed deer (O. h. sitkensis) and white-tailed deer were analysed electrophoretically for serum albumin phenotype with methods adapted from McClymont et al. (1982). For each deer 0.5 g tissue, usually skeletal muscle, was ground in 1 milliliter (ml) distilled water, centrifuged ten min. at 12,000 x gravity (g) and 50 microliters (ul) of the supernatant mixed with 5 ul bromphenol blue and one drop of glycerol before loading 5-30 ul onto the gel. Gels were 6-15% linear gradient polyacrylamide with a 5% stacking gel. The gel buffer was 1.5 M tris-HCl, pH 8.8 and the electrode buffer was 0.2 M tris, 1.5 M glycine-HCl, pH 8.3. Vertical electrophoresis was done at 100-200 volts for 5-12 hours. Ice packs were laid against the gel plates during electrophoresis to prevent overheating. Gels were stained in 0.1% Coomassie blue, 10% acetic acid, 45% methanol (wt/vol/vol). Destaining was in 10% acetic acid, 45% methanol initially, followed by several washes in 7% acetic acid.

Table 1. Species, subspecies and locations sampled.

Species		Location
White-tailed deer	(O. v. virginianus)	Clarke Co. Georgia
"	(O. v. borealis)	Montgomery Co. Ill.
"	(O. v. dakotensis)	Montana (eastern)
"	(O. v. ochrourus)	Montana (western)
Mule deer	(O. h. hemionus)	Montana/Wyoming
Black-tailed deer	(O. h. sitkensis)	Kodiak Is. Ak.
Elk	(C. e. nelsoni)	Montana
Pronghorn	(A. americana)	Montana
Plains bison	(B. b. bison)	various herds see table 3
Wood bison	(B. b. athabascae)	Elk Is. Nat. Park, Alberta
Presumptive p. bison-w.bison hybrids		Northwest Terr. Canada
Cattle various breeds	(Bos taurus)	Bozeman, Mt.
Black bear	(U. americanus)	Montana
Grizzly bear	(U. arctos)	Montana

Mitochondrial DNA purification

For mtDNA analysis brain tissue was found to be far superior to other tissues for ease of homogenization and digestion of DNA. Liver and kidney gave good yields of mtDNA which was sometimes difficult to digest. Skeletal muscle yielded lower but adequate amounts of mtDNA.

Fresh tissue was best, but frozen samples also gave good results if stored less than two months.

Standard procedures for extraction of mtDNA were adapted from Powell and Zuñiga (1983) and Davis et al. (1980) and are described in Appendix A.

For some of the liver and kidney samples stored frozen for extended periods purification in cesium chloride (CsCl) gradients was necessary to allow digestion with endonucleases. Isolation and lysis of mitochondria were done as in steps 1-10 of the standard procedures in Appendix A. The procedures of Lansman et al. (1981) for CsCl gradient centrifugation were then followed. Granular CsCl (1.1 g) was added to each ml of lysate along with 0.2 ml of a 10 mg/ml ethidium bromide solution. This mixture was put into an ultracentrifuge tube and mineral oil added to the top of the tube. After balancing paired tubes to 0.01 g samples were centrifuged at 160,000 x g for 48 hrs. After centrifugation the visible band of mtDNA was removed from the tube with a hypodermic needle. Ethidium bromide was removed by extraction with water-saturated 1-butanol (Maniatis et al., 1982) and the sample volume increased 1.5 times by adding water. Precipitation and resuspension of mtDNA was done as in steps 15-19 of the standard purification procedures.

Digestion and identification of mtDNA fragments

Six to eighteen ul of solution containing approximately 0.3 ug purified mtDNA were put into a 1.5 ml Eppendorf tube with 2 ul reaction buffer, 1 ul RNase-A (10 mg/ml stock), 1 ul restriction

enzyme and sterile water to a volume of 20 ul. The reaction buffers used were ten times the required concentration, so that 2 ul added to a 20 ul mixture resulted in the proper buffer concentration. Different restriction enzymes require different buffers, specified by the manufacturer or Maniatis et al. (1982). Restriction enzymes were obtained from New England Biolabs and Boehringer Mannheim Biochemicals. Digestion reactions were carried out at 37C for 4-14 hrs. The enzymes used are listed in Table 2 on page 17.

Eight to twelve ul of the digestion mixture was applied to 0.65% (6-base enzyme digests) or 1.2% (4-base enzyme digests) horizontal agarose gels. Gel and electrode buffer was 89 mM tris, 89 mM boric acid, 2 mM EDTA, pH 8.0 (Maniatis et al., 1982). Electrophoresis was done at 20-50 v for 5-16 hrs. Gels were stained in 0.5 ug/ml ethidium bromide in electrode buffer for 5- 30 min., visualized under shortwave ultraviolet light and photographed with Polaroid type 55 film through a red gelatin filter.

For each gel, fragment sizes were estimated by comparison with standards of known size (Lambda virus DNA digested with Hind III or Bam HI). The migration distances of the standards were measured and plotted on semilog graph paper with migration distance on the x axis and fragment size (bp) on the logarithmic y axis. Linear duplex DNA molecules travel through gels at rates approximately inversely proportional to the $\log_{10}$ of their molecular weights so that the log molecular weight (or log number base pairs) versus migration distance in agarose gels is approximately an inverse linear relationship (Maniatis et al., 1982). The migration distances for the fragments

of digested mtDNA were then measured and their sizes estimated from the graph.

Estimation of genetic distance and divergence time

Fragments were judged to be homologous between two organisms if migration distances appeared identical in side by side comparisons on a gel. The proportion of shared (homologous) fragments was calculated for each pair-wise comparison using equation 21 of Nei and Li (1979):

$$F = 2N_{xy}/N_x+N_y$$

where N_x and N_y are the numbers of fragments from all digestions in organisms x and y respectively and N_{xy} is the number of fragments shared by the two organisms. The number of base substitutions per nucleotide, p, was estimated using equation 6b of Upholt (1977):

$$p = 1- \left[\frac{-F+ (F^2 + 8F)^{1/2}}{2}\right]^{1/n}$$

where n equals the number of base pairs recognized per cleavage site.

For the comparisons between deer groups and between bison and cattle, p was calculated separately for the 6 (n=6) and 4 (n=4) base enzyme assays, and a weighted average was used as a final p value using the equation:

$$p = \frac{p_1N_1 + p_2N_2}{N_T}$$

where p_1 and N_1 are the p values and number of fragments for 6-base

enzyme assays respectively and p_2 and N_2 are similar values for 4-base enzyme assays. N_T is the total number of fragments for all digests.

For some of the species, estimates of divergence time (time since two organisms last shared a female ancestor) were made using an estimated mtDNA sequence divergence rate of 2-4% per million years (Brown et al., 1979; Ferris et al., 1983a, 1983b) and the equation:

$$\text{divergence time} = \frac{p}{.02 \text{ (or } .04) \times 10^{-6}}$$

Samples analysed

Inter-species comparisons

At least one individual of each species (pronghorn, elk, white-tailed deer, mule deer, bison, and cattle) was analysed with a battery of ten 6-base enzymes. Also some cattle, bison, mule deer and white-tailed deer were analysed with two four-base enzymes. One black bear and one grizzly bear were screened with six six-base enzymes. The samples analysed are given in Tables 2 and 3 on pages 17 and 19.

Mule deer-white-tailed deer comparisons

All deer sampled were subject to albumin electrophoresis. In addition a total of 15 Montana mule deer, one Wyoming mule deer, one Alaska black-tailed deer, 19 Montana white-tailed deer, 5 Georgia white-tailed deer and 4 Illinois white-tailed deer were analysed with two enzymes, Eco RI, and Hae III which gave polymorphic profiles among deer.

RESULTS

Comparison of all species

Fragment Patterns

Table 2 shows the mtDNA fragment patterns resulting from restriction enzyme digestions. A representative electrophoretic gel, Figure 1, contains the fragment patterns resulting from digestion of deer and elk mtDNA with the enzymes Cla I and Sac I. For several of the enzymes the patterns are similar to those reported by Laipis et al. (1979) for cattle and Carr (personal communication) for the two deer species. Pronghorn, white-tailed deer and mule deer showed intra-specific variability. Only digests with Bgl II resulted in variable patterns in pronghorn, shown in Table 2. No variability was observed in bison from several herds (Table 3). The variable digestion patterns for deer are shown in Table 4, and composite deer mtDNA phenotypes are given in Table 5.

From Table 5 it can be seen that the composite phenotypes consist of combinations of the variable digestion patterns shown in Table 4. For example, composite type A consists of Eco RI pattern 1, Xba I pattern 1, Cla I pattern 1 and Hae III pattern 2. All enzymes except those listed in Tables 4 and 5 resulted in invariant patterns among both mule deer and white-tailed deer, shown in Table 2.

Figure 1. Photograph of Sac I and Cla I digestion patterns on a 0.65% agarose gel stained with ethidium bromide.

Lanes A-D, F-I, white-tailed deer, Cla I,

(Lane B incomplete digestion)

Lane E, mule deer, Cla I

Lane L, elk, Sac I

Lane M-P, white-tailed deer, Sac I

Lane Q, mule deer, Sac I

Lane J, size standard, Lambda virus DNA digested with Hind III

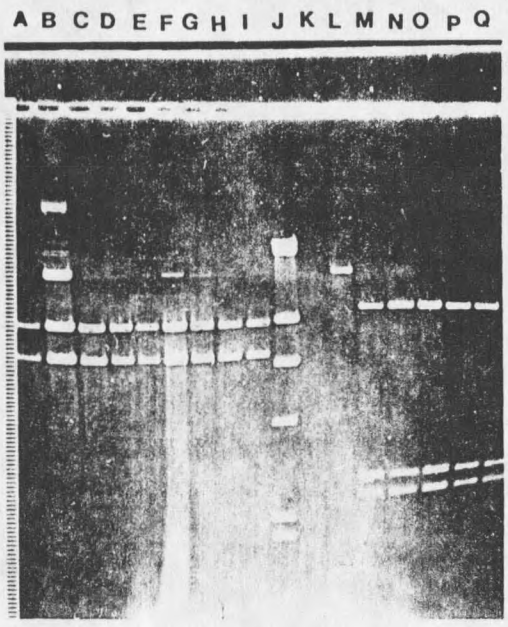


Table 2. Mitochondrial DNA restriction fragment patterns.[1]

Enzyme	Species						
A. Six-base enzymes							
	Deer	Elk	Prong-horn	Bison	Cattle	Black Bear	Grizzly Bear
Eco RI	8.9(18) 3.1(26) 1.9 1.7	12.0(6) 3.1	8.4(4) 7.6	7.3[2] 4.6(9) 4.2	7.3(8) 4.6 4.2	4.8(1) 3.6 3.2	16.6(1)
Bam HI	7.5(10) 5.3(19) 3.8	9.3(4) 5.3 2.4	7.4(4) 4.5 3.8	5.9[2] 5.2(6) 3.7	11.0(4) 3.4 2.0	15.0(1) 2.0	9.4(1) 7.0
Hind III	10.5(10) 3.9(17) 1.6 .8	11.5(6) 4.2 1.6	16.6(4)	15.0[2] 1.6(7)	10.5(7) 4.6 1.6	7.5(1) 2.7 1.9 1.7 1.5	3.2(1) 2.7 2.0 1.9 1.5
XbaI	7.6(4) 5.2(12) 3.2	10.5(4) 3.2 2.6	13.0(3) 3.2	6.5[2] 3.7(3) 3.0 2.6	4.8(4) 3.2 3.0 2.5 1.8	5.6(1) 3.4 3.2	5.6(1) 3.4 3.2
Bgl II	16.6(2) (8)	11.5(3) 2.6 1.2	9.8(1) 6.6 or 16.6(3)	9.8(1) 6.6	9.8(4) 6.6	9.0(1) 7.2	10.5(1) 5.9
Pvu II	16.6(7) (16)	13.0(4) 3.5	6.1(3) 4.6 3.1 2.8	14.0[2] 2.7(4)	14.0(4) 2.7	8.6(1) 6.2	10.0(1) 6.6
ClaI	9.0(6) 7.1(14)	9.0(3) 7.1	7.2(3) 6.0 1.9 1.5	7.2(2) 6.4 1.6	16.6(2)		

Table 2. continued

	Deer	Elk	Prong-horn	Bison	Cattle	Black Bear	Grizzly Bear
BclI	8.0(4) 3.6(7) 3.0 .9	4.8(1) 4.6 3.5 2.7	4.0(1) 3.0 2.5 2.0 1.8 1.2	12.0(2) 4.0	11.0(2) 6.0		
SacI	10.0(6) 3.1(14) 2.7	16.6(4)	16.6(3)	11.0[2] 2.8(4) 2.3	16.6(3)		
PstI	No (3) cuts(3)	16.6(1)	16.6(3)	No (1) cuts	9.4(4) 6.3		

B. Four-base enzymes

	Deer	Bison	Cattle
Hae III	4.0(18) 2.6(26) 1.2 1.1	2.0(2) 1.5 1.3 1.1 .8	2.0(2) 1.5 1.0 .8
Sau 96I	3.5(6) 3.2(7) 2.0 1.5 1.2 .8	5.6(2) 3.5 1.6	4.0(2) 2.9 2.2 1.8 1.6 1.0

[1]Numbers refer to size of fragments (kilobases) generated by enzyme digestion. Numbers in parentheses refer to number of animals yielding a particular pattern. For deer the upper number in parentheses is mule deer, the lower white-tailed deer. Note there are two patterns for pronghorn mtDNA digested with BGL II. Only the most common patterns for both deer species are given here, variable patterns and composite mtDNA phenotypes for populations and species of deer are given in table 4.

[2]Indicates sample includes wood bison.

Table 3. Numbers of bison from various herds analysed with different restriction enzymes.[1]

	Herd				
Enzyme	EINP[2]	NWT[2]	YNP[2]	HM[2]	NB[2]
EcoRI	1	1	3	4	
BamHI	1	1	3	1	
HindIII	1	2	2	1	1
XbaI	1			1	1
BglII		1			
PvuII	1	2			1
ClaI		1		1	
BclI		2			
SacI	1	2			1
PstI				1	
HaeIII		2			
Sau96I		2			

[1]Fragment patterns for all digestions are as in table 2, there is no inter-herd variation.

[2]EINP = Elk Island National Park, Alberta (wood bison), NWT = Northwest Territories, Canada, (presumed wood bison-plains bison hybrids), YNP = Yellowstone National Park (plains bison), HM = Henry Mountains, Utah (plains bison) and NB = Nielson herd, Alberta (plains bison). Blank spaces indicate no analyses done.

Table 4. Variable mtDNA digestion patterns in deer.[1]

Enzyme										
EcoRI			XbaI		ClaI		HaeIII			
1	2	3	1	2	1	2	1	2	3	4
8.9	8.9	7.0	7.6	13.1	9.0	16.6	4.0	4.0	4.0	3.0
3.1	3.6	5.0	5.2	3.2	7.1		2.6	1.1	1.3	1.5
1.9	3.1	4.7	3.2				1.2	1.0	1.1	1.2
1.7							1.1		1.0	

[1]Numbers indicate fragment size in kb.

Table 5. Composite mtDNA phenotypes in deer.[1]

Population	N[2]	Enzyme				Composite Pattern
		EcoRI	XbaI	ClaI	HaeIII	
MtMD[2]	2	1	1	1	2	A
MtMD[2]	2	2	1	1	1	B
GaWT[2]	3	2	1	1	1	B
MtWT[2]	4	1	1	1	1	C
IlWT[2]	2	1	1	1	1	C
MtMD[2]	1	1		1	3	D
GaWT[2]	1	2	2	1	1	F
GaWT[2]	1	1	1	2	1	G
AkBT[2]	1	3			4	E

[1]The numbers refer to the digestion patterns in Table 4. Blanks in the table indicate no analyses done.

[2]N=number of animals, Mt=Montana, Ga=Georgia, Il=Illinois, Ak=Alaska, MD=mule deer, WT=white-tailed deer, BT=black-tailed deer.

Divergence estimates

Table 6 shows estimates of genetic distance between taxa, expressed as the number of base substitution per nucleotide (p) from my analysis and those reported elsewhere. Table 7 shows divergence time of several taxa estimated from p values and the fossil record.

Table 6. Estimated genetic distance in base substitututions per nucleotide (p) for mtDNA.

Organisms	No. Enzymes	p	Standard[5] Deviation	Source
Intra-species:				
MtWTxI1WT[1]	12	.0000		
MtWTxGaWT[1]	12	.0025- .0053[3]	.0036- .0051	
GaWTxGaWT[1]	12	.0026[4]-.0084[3, 4]	.0037- .0063	
MtMDxMtMD[1]	12	.0066[4]	.0057	
Pronghorn	10	.0028[4]	.0038	
WBxPB[1]	6	.0000		
Humans	7	.0036		(Brown, 1980)
Peromyscus spp.	6	.0050[4]-.0150		(Avise et al., 1979a)
Lepomis macrochiris	13	.0870		(Avise et al., 1984)
Intra-family, inter-species				
MtWTxMtMD[1]	12	.0025- .0040[3]	.0036- .0045	
MtMDxGaWT[1]	12	.0000- .0095[3]	.0068	
CaMDxSCWT[1]	10	.0720		(Carr, personal communication)
BBxGB[1]	6	.0580	.0190	
Peromyscus maniculatus P. polionotus	6	.1300- .1700		(Avise et al., 1979a)
CattlexBison	10	.0614	.0141	
Elkxdeer[2]	10	.0850	.0170	
GoatxSheep	2	.0600- .1100		(Upholt and Dawid, 1977)

Table 6 continued

Organisms	No. Enzymes	p	Standard[5] Deviation	Source
Intra-order, inter-family				
DeerxPH[1, 2]	10	.1130- .1330[3]	.0189- .0198	
ElkxPH	10	.1300- .1310[3]	.0200- .0199	
BisonxPH	10	.1130- .1558[3]	.0188- .0214	
CattlexPH	10	.1130- .1558[3]	.0188- .0214	
Deerxbison[2]	10	.1980	.0235	
Deerxcattle[2]	10	.1287	.0202	
Elkxbison	10	.1531	.0217	
Elkxcattle	10	.1274	.0203	
Humanxbaboon	11	.2300- .2900		(Brown et al., 1979)

[1]PH=pronghorn, PB=wood bison, WB=wood bison, BB=black bear, GB=grizzly bear, Ca=California, SC=South Carolina. Other abbreviations as in Table 5.

[2]For inter-species comparisons with deer the most common 6-base patterns (Table 2) were used.

[3]Ranges of values are due to intra-group variation and reflect differences between composite types in Table 5 for deer and the two BglII patterns for pronghorn.

[4]These values are for comparisons within a limited geographic area.

[5]Standard deviations calculated as in Upholt (1977).

Table 7. Divergence times calculated from p values and estimated from the fossil record.

Taxa	Divergence time (MYBP)		
	derived from p[1]	fossil record	Source
Cervidae-Antilocapridae	2.83-6.65	20-25 Miocene	(Romer, 1966)
Bovidae-Antilocapridae	2.83-7.79	15-20 Miocene	(Romer, 1966) (Kurten, 1972)
Bovidae-Cervidae	3.19-9.90	20-25 Miocene	(Romer, 1966)
Cervus-*Odocoileus*	2.13-4.25	15-25 Miocene	(Scott, 1937) (Osborn, 1910) (Geist, 1981)
Bos-*Bison*	1.54-3.07	1-3 Pliocene-Pleistocene	(McDonald, 1981; Kurten, 1968)
Ursus arctos-*U. americanus*	1.45-2.90	1-2 Pleistocene	(Herrero, 1972; Kurten and Anderson, 1980)
Odocoileus virginianus-*O. hemionus* (California)	1.80-3.60 (Carr, personal communication)	0.5-2 Pleistocene	(Kurten and Anderson, 1980; Geist, 1981)
O. virginianus-*O. hemionus* (Montana)	0.48-0.60	0.5-2 "	

[1]Assuming a rate of mtDNA divergence of 2-4% per million years.

Mule deer-white-tailed deer comparisons

Serum albumin phenotyping

The results of albumin electrophoresis are presented in Table 8. Except for five heterozygotes (2%) all whitetails and mule deer displayed the species-specific albumin band as reported by McClymont et al. (1982). Black-tailed deer had the mule deer-typical band. Figure 2 is a photo of the different patterns.

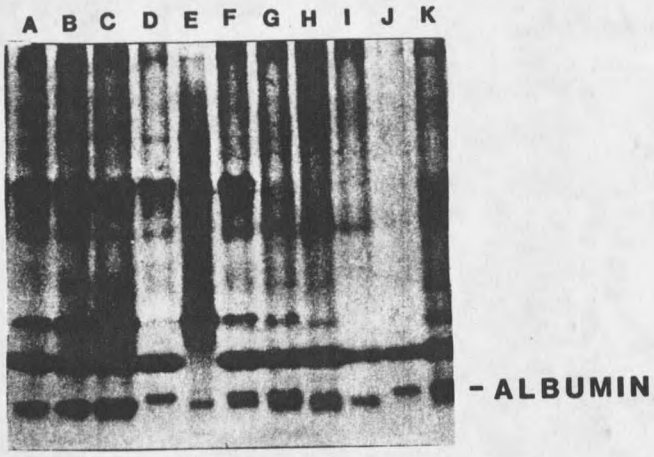
Table 8. Numbers of morphological and serum albumin types of deer from all sampled locations.

		Morphological type			
		Mule deer	White-tailed deer	Black-tailed deer	Total
Albumin genotype	FF[1]	93	0	3	96
	SS	0	162	0	162
	FS[2]	2	3	0	5
Total		95	165	3	263

[1]F = fast, S = slow allele.

[2]All Heterozygotes were from Montana.

Figure 2. Photograph of polyacrylamide gel showing serum albumin bands for mule deer (lanes A, B, C, E, H, I), white-tailed deer (lanes D, K) and heterozygotes (lanes F, G). Lane J shows albumin from a deer with intermediate morphological characters and the whitetail-typical albumin band (see discussion).



Mitochondrial DNA analysis

Two enzymes, Eco RI and Hae III, provided digestion patterns which varied between individual deer in Montana. The digestion profiles for the ten other enzymes were virtually identical in all deer of both species from all locations. Besides Eco RI and Hae III the only variable profiles in deer resulted from Cla I and Xba I digestions in Georgia whitetails (Table 4).

Figure 3 is a representation of the polymorphic digestion profiles in the combinations found in individual deer. Note that types A-D consist of two Eco RI and three Hae III patterns found in four combinations. Type E, observed only in the Sitka blacktail, consists of two unique patterns. These types, A-E, represent the same types identified in Table 5 except only two of the variable enzymes were used here. Since mtDNA is presumably inherited as an intact unit from the mother, each individual's profile constitutes a "type", indicative of maternal ancestry. Table 9 shows the distribution and locations of mtDNA, albumin and morpholgical types while Figure 4 shows the geographic location of types in Montana.

Several things are apparent from these data. First, in Montana the mule deer are relatively variable, exhibiting three mtDNA types, A, B or D while only type C is seen in whitetails. Second, in Montana deer there is species-specificity in morphology, serum albumin (except heterozygotes) and mtDNA. Third, the Sitka blacktail mtDNA is very different from that of Montana mule deer and whitetails.

Despite the distinctions between these mtDNA digestion profiles for two enzymes, the overall similarity of profiles for mule deer and whitetails for the entire 12 enzyme battery is striking. From Table 6 it can be seen that the p value for comparisons of Montana mule and white-tailed deer is as low as those for intra-species comparisons of other taxa.

Figure 3. Representation of agarose gels containing composite mtDNA digestion profiles for EcoRI and HaeIII for deer. Fragments less than 1000 base pairs could not be consistently identified and are not included. Fragment sizes (kilobases) are listed below each pattern.

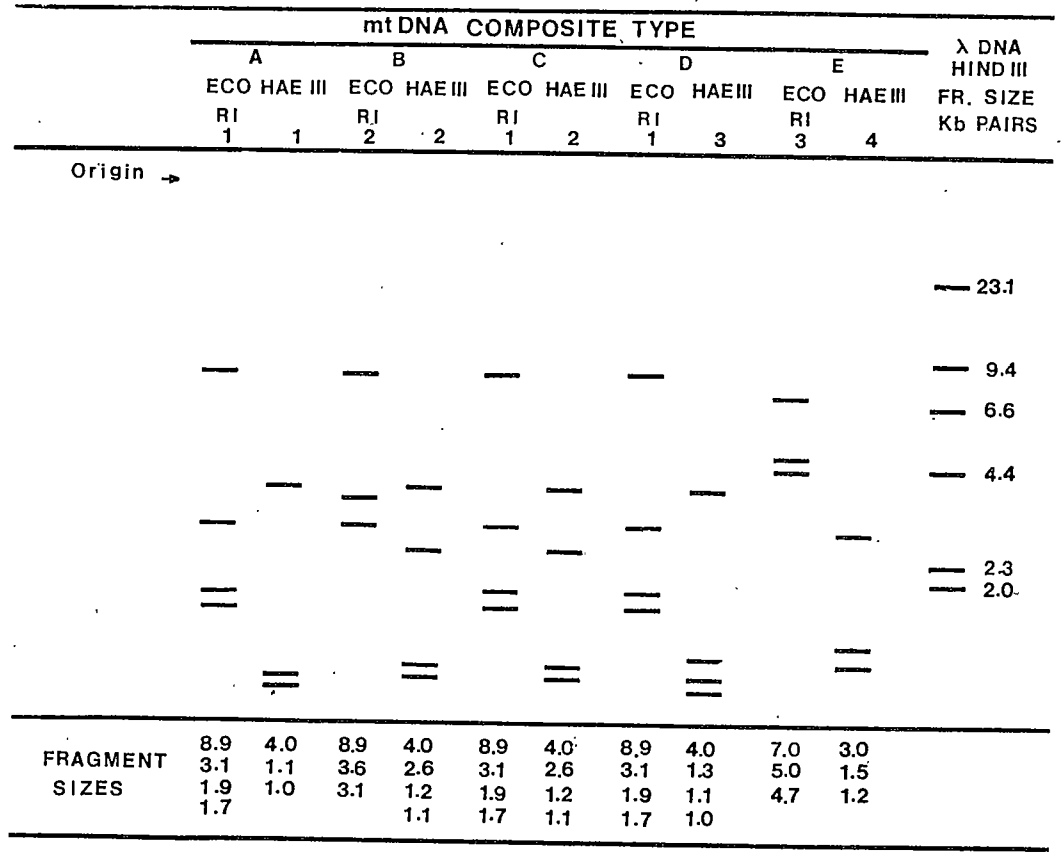
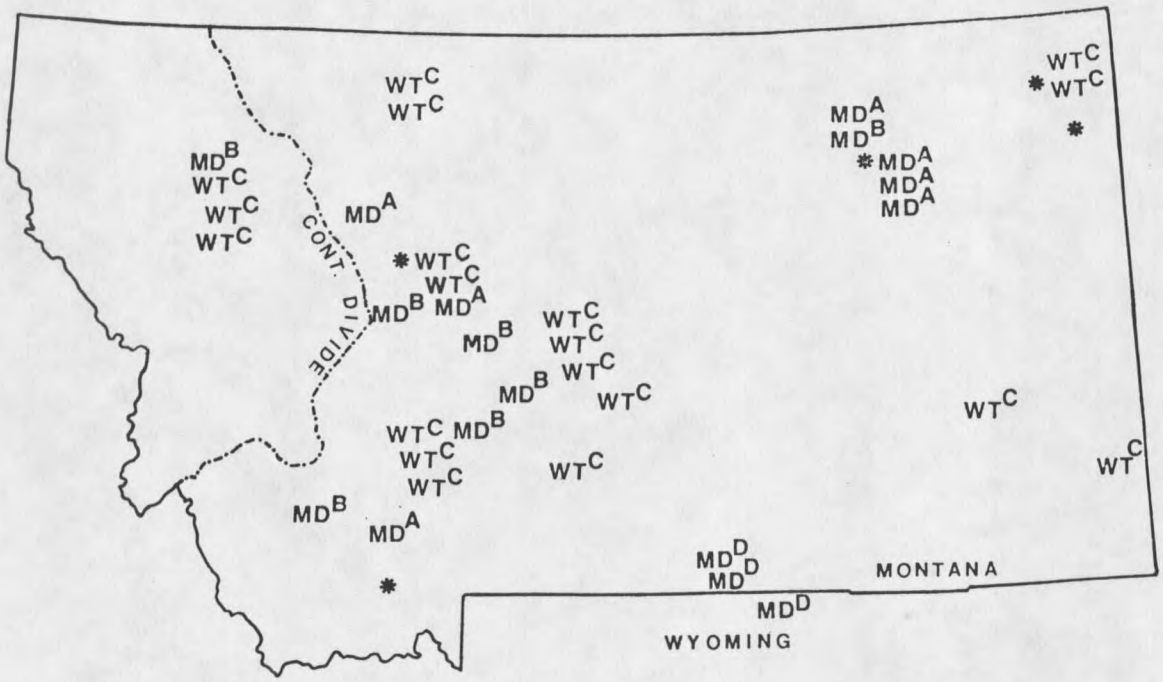


Table 9. Morphological, serum albumin and mtDNA types for deer.

morphology specimen No.	albumin type	mtDNA type	location
mule deer no. 1-6	FF	A	Montana
mule deer no. 7	FS	A	Montana
mule deer no. 8-13	FF	B	Montana
mule deer no. 14-16	FF	D	Bighorn/Pryor Mts. Montana, Wyoming
whitetail no. 1-17	SS	C	Montana
whitetail no.18-19	FS	C	Montana
whitetail no. 20-23	SS	B	Clarke Co. Ga.
whitetail no. 24	SS	C	Clarke Co. Ga.
whitetail no. 25-28	SS	C	Montgomery Co. Ill.
blacktail no. 1	FF	E	Kodiak Is. Ak.

Figure 4. Geographiic distribution of mtDNA types for individual deer. Two letter abbreviations refer to morphological type, md=mule deer, wt=whitetail, followed by the letter designation of the mtDNA type from Figure 3. An asterisk in front of the letters indicates that animal is an albumin heterozygote. The two asterisks without letters represent heterozygotes not analysed for mtDNA.



DISCUSSION

The data presented in Tables 6 and 7 show that divergence among taxa (as indicated by traditional taxonomic groupings or divergence times from the fossil record) is accompanied by mtDNA sequence divergence (as indicated by the estimated base substitutions per nucleotide, p). Inter-family comparisons yield the highest estimates of mtDNA sequence divergence, followed by intra-family-inter-species and intra-species comparisons.

This relative ordering of divergence values is expected if one assumes a constant rate of random nucleotide substitution by mutation. However, estimates of genetic divergence using comparisons of fragments from mtDNA digestion profiles have been found to be most accurate when at least 20% of the fragments are homologous (F is greater than 0.20 (Upholt, 1977)) or when p is less than 0.05 (Ferris et al., 1983b). With decreasing values of F the chance of incorrectly scoring fragments as homologous increases, which leads to a proportional underestimate of p (Brown et al., 1979). Also, some cleavage sites seem to be highly conserved in evolution, implying that not all sites on the mtDNA molecule are equally susceptable to mutational change. The presence of conserved sites will cause furthur underestimation of p (Brown et al., 1979). These authors suggest that p values are most accurate for lineages separated within the last 5 million years while those for lines separated by 25 million years are underestimated by about 50%.

Another factor which may cause underestimation of divergence is apparent homology between restriction sites (or fragments) resulting from convergent gain or loss of sites, not common ancestry (Templeton 1983). Given these considerations, the underestimation of divergence times using p values for inter-family comparisons (Table 7) is not surprising. Other techniques, such as DNA-DNA hybridization (Sibley and Ahlquist, 1983, 1984) may be more suitable for obtaining estimates of nucleotide sequence divergence for such distantly related taxa.

Intra-specific comparisons

The estimated genetic distance between conspecifics in Table 6 range from 0.0000 to 0.0870. Elk and cattle, not listed in the intraspecific comparisons, displayed no mtDNA variation between conspecifics (p=0).

The lower of the two p values for intraspecific comparisons of Peromyscus spp. in Table 6 was derived from comparisons of individual mice within local populations and the higher value from comparisons of geographically separated populations (Avise et al., 1979a). Avise et al. (1979b) obtained similar low p values for local and high p values for geographically separated populations of Geomys pinetis. For humans, p was derived from comparisons of several individuals of different races (Brown, 1980) and for Lepomis macrochirus from comparisons of two distinct subspecies (Avise et al., 1984).

Intraspecific variation within limited geographic areas appears to be similar in all species in Table 6 (p=0.0026-0.0084). It appears, however, that large mammals (deer, bison and humans) exhibit

less intra-specific variation in mtDNA (p=0.0000-0.0053) between subspecies and geographic areas than rodents and fish (p=.015-0.087). The analyses of deer and bison mtDNA illustrate this point. Wood bison and plains bison are considered morphologically distinct subspecies (Geist and Karsten, 1977; McDonald, 1981). My analysis of mtDNA and other work with blood groups (Peden and Kraay, 1979), blood proteins (D. Yardley, personal communication), and karyotypes (Ying and Peden, 1977) failed to detect differences between the subspecies. As with bison, deer showed little or no sub-specific variation in mtDNA (Tables 4, 5 and 6). This conservation of mtDNA sequence between geographically separated populations of artiodactyls may be due to extensive mitochondrial gene flow, selection for certain mtDNA sequences or relatively recent founding events and short divergence times. The situation is apparently different in the geographically variable rodents and fish.

Intra-family, inter-species comparisons

Except for comparisons of Montana mule deer with white-tailed deer the p values for intra-family, inter-species comparisons in Table 6 are intermediate to the intra-species and inter-family values and generally consistent with data from other studies. The estimated divergence times for these groups (Table 7) are likewise in general agreement with the fossil record except for the deer-elk comparison. Osborn (1910), Scott (1937) and Geist (1981) note that the subfamilies Cervinae and Odocoilinae, represented by *Cervus* and *Odocoileus* respectively, may have been separate lineages since the Miocene or

earlier. Baccus et al. (1983) using protein electrophoresis obtained relative genetic distance values between cervids indicative of this subfamilial relationship. This would indicate our estimates of divergence, p, and divergence time are underestimates as with the inter-family comparisons. Nonetheless, our p value indicates that these two deer species are more closely related to each other than to members of the other families. Since the fossil record is not definitive regarding the origin of these subfamilies furthur analysis of nuclear and mitochondrial DNA may clarify the degree of divergence of these groups.

Stormont et al. (1961) proposed that bison be included in the same genus as cattle, Bos, due to similarities of blood groups. Baccus et al. (1983), reporting similarity of proteins detected by electrophoresis, agreed with this suggestion. The p value obtained in this study for these two species is comparable to those from other congeneric comparisons (Table 6) but more inter-genus comparisons (in addition to deer-elk) are needed to establish criteria for classification.

Mule deer-white-tailed deer comparisons

A lack of quantitative measurement of morphological characters used to distinguish the species led us to categorize deer by the collector's judgement. None of the five albumin heterozygotes were reported to have intermediate morphology. For some other deer intermediate characters were reported (eg. a whitetail with large

ears, or a mule deer with whitetail type antlers). In all cases but one these animals gave albumin patterns in agreement with the overall morphological species designation. The one exception was a male described as having whitetail antlers, head and running gait, mule deer rump and tail and was associated with a group of whitetail does. Unfortunately, no mtDNA analysis was possible but he gave the typical whitetail albumin pattern. This deer was excluded from the albumin data in Table 1 since he did not fit either morphological category.

Deer with intermediate morphology are periodically reported by wildlife biologists and hunters in Montana (Mackie, personal communication). These deer, the apparent species-specificity of serum albumin and occurrence of rare heterozygotes suggests that limited hybridization occurs. Since there was no indication of intermediate morphology in the albumin heterozygotes it cannot be determined if they resulted from recent hybridization or introgression of the rare allele into each species in the more distant past. Since mtDNA is maternally inherited, heterozygote mule deer no. 7, with mule deer specific type A mtDNA, has probable mule deer female ancestry and whitetail paternal influence, while whitetail heterozygotes nos. 18 and 19, with whitetail specific type C mtDNA, are in a whitetail female lineage with mule deer paternity in their past. It appears that hybridization between these two species may involve either sex.

The mtDNA data indicate that Montana mule deer consist of at least three maternal lines, types A and B appear to be intra-populational variants while type D may represent a lineage isolated from other sampled populations. Montana whitetails are in a fourth

distinct lineage. These data suggest that at present, if F-1 hybrid females breed at all, they will breed back to the mother's species, keeping the maternal lineage species-specific.

Despite these distinctions the overall similarity of mtDNA types between the deer species in Montana, and the dissimilarity between Montana mule deer and Sitka blacktails' mtDNA require explanation. Blacktails and mule deer, both subspecies of O. hemionus should be more genetically similar to each other than either is to O. virginianus. The data for serum albumin support this while the mtDNA data do not. Carr (personal communication) reports digestion profiles of Texas mule deer and whitetails and South Carolina whitetails similar to my samples from Montana, Illinois and Georgia deer. California mule deer are quite different for a ten enzyme battery, with an Eco RI pattern similar to my Sitka blacktail. If the mtDNA of the Sitka blacktail and California mule deer represents the ancestral O. hemionus type and that in eastern whitetails represents the ancestral O. virginianus type, then the Montana (and Texas) mule deer actually have the whitetail type. A plausible explanation for this suggested by Carr (personal communication) is introgression of mtDNA following hybridization. Other examples of possible inter-species or inter-subspecies transfer of mtDNA in animals are reported by Ferris et al. (1983a), Powell (1983), Avise et al. (1984) and Spolsky and Uzzell (1986). If introgression of whitetail mtDNA into mule deer has occurred in the past our observations suggest that in Montana some divergence has occurred between the species' mitochondrial genomes since the initial hybridization event(s).

The data seem to support the sympatric theory of origin of isolating mechanisms (Mayr, 1970) which postulates that following geographic speciation, incipient species will at first hybridize freely. This is followed by reduced levels of hybridization after selection against hybrids results in effective reproductive isolating mechanisms. O. hemionus and O. virginianus probably speciated in the Pleistocene (Kurten and Anderson, 1980) perhaps during geographic separation due to continental glaciers. A relatively high level of hybridization between the species after contact was re-established could have resulted in the apparent introgression of mtDNA from whitetails into mule deer. Subsequent character displacement of behavior, morphology and habitat requirements, the divergence of nucleotide sequences and reduced levels of hybridization between the species may account for the species-specificity of these traits in extant populations in Montana.

Perhaps mule deer populations were initially established by males dispersing into unoccupied habitat and breeding with resident whitetail females. The F-1 hybrid females may have successfully bred back to mule deer males establishing a population with mule deer morphology and whitetail mtDNA. The apparent fixation of the whitetail type mtDNA in mule deer populations may be due to random drift in small founding populations, but the widespread occurrence of this phenomenon may demand another explanation. Furthur study of mitochondrial and nuclear genomes, behavior of sympatric and allopatric populations, controlled breeding and reconstruction of the

species' phylogenies from the fossil record are needed to fully explain the distribution of mtDNA reported here.

CONCLUSIONS

The results from intra- and inter-species comparions indicate that:

1. mtDNA restriction fragment analysis may result in underestimates of divergence for inter-family comparisons of artiodactyls.
2. Estimates of base substitutions per nucleotide, p, for intra-species comparisons of artiodactyls are generally 0.000 to 0.008 while intra-family, inter-species comparisons result in p values of 0.05 to 0.09.
3. Estimates of divergence times from p values and the fossil record are similar for species (black bear-grizzly bear, cattle-bison) separated 1 to 3 mybp.

The results from comparisons of white-tailed and mule deer indicate that:

1. Limited hybridization probably occurs between the deer species in Montana, both sexes of both species may be participating.
2. mtDNA, serum albumin and morphology appear distinct between Montana mule and white-tailed deer, suggesting that inter-species gene flow is not extensive at present. The apparent species-specificity of mtDNA suggests that F-1 hybrid females breed back to the mothers' species if at all.

3. There is apparently limited mitochondrial gene flow between mule deer from the Bighorn/Pryor Mountains area and other sampled locations.
4. mtDNA in both deer species in Montana and Wyoming is very similar to that of eastern whitetails, and different from Sitka blacktails suggesting introgression of the whitetail type into mule deer populations following hybridization at some time in the past.

LITERATURE CITED

ANDERSON, S., A. T. BANKIER, B. BARRELL, M. de BRUIJN, A. COULSON, J. DROUIN, I. EPERON, D. NIERLICH, B. ROE, F. SANGER, P. SCHREIER, A. SMITH, R. STADEN AND I. YOUNG. 1981. Sequence and organization of the human mitochondrial genome. Nature 290:457-465.

_____, M. de BRUIJN, A. COULSON, I. EPERON, F. SANGER AND I. YOUNG. 1982. Complete sequence of bovine mitochondrial DNA. J. Mol. Biol. 156:683-717.

AVISE, J. C., R. A. LANSMAN AND R. O. SHADE. 1979a. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. I. Population structure and evolution in the genus Peromyscus. Genetics 92(1):279-295.

_____, C. GIBLEN-DAVIDSON, J. LAERM, J. PATTON AND R. LANSMAN. 1979b. Mitochondrial DNA clones and matriarchal phylogeny within and among geographic populations of the pocket gopher, Geomys pinetis. Proc. Natl. Acad. Sci. USA 76:6694-6698.

_____, E. BERMINGHAM, L. G. KESSLER AND N. C. SAUNDERS. 1984. Characterization of mitochondrial DNA variability in a hybrid swarm between subspecies of bluegill sunfish. Evolution 38:931-941.

BACCUS, R., N. RYMAN, M. H. SMITH, C. REUTERWALL AND D. G. CAMERON. 1983. Genetic variability and differentiation of large grazing mammals. J. Mamm. 64(1):109-120.

BAKER, R. H. 1984. Origin, classification and distribution. Pages 1-18 in L. K. Halls, editor. White-tailed deer, ecology and management. Stackpole books, Harrisburg, PA.

BIBB, M., R. VAN ETTEN, C. WRIGHT, M. WALBERG AND D. CLAYTON. 1981. Sequence and gene organization of mouse mitochondrial DNA. Cell 26:167-180.

BROWN, G. G. AND M. V. SIMPSON. 1981. Intra- and interspecific variation of the mitochondrial genome in Rattus norvegicus and Rattus rattus: Restriction enzyme analysis of variant mitochondrial DNA molecules and their evolutionary relationships. Genetics 97:125-143.

BROWN, W. 1980. Polymorphism in mitochondrial DNA of humans as revealed by restriction endonuclease analysis. Proc. Natl. Acad. Sci. USA 77:3605-3609.

BROWN, W., M. GEORGE JR. AND A. WILSON. 1979. Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. USA 76:1967-1971.

CHESSER, R. K. C. REUTERWALL AND N. RYMAN. 1982. Genetic differentiation of Scandinavian moose Alces alces populations over short geographical distances. Oikos 39:125-130.

COOTE, J., GY. SZABADOS AND T. WORK. 1979. The heterogeneity of mitochondrial DNA in different tissues from the same animal. FEBS Letters 99(2):255-260.

DAVIS, R. W., M. THOMAS, J. CAMERON, T. P. ST. JOHN, S. SCHERER AND R. A. PADGETT. 1980. Rapid DNA isolations for enzymatic and hybridization analysis. Methods Enzymol. 65:404-411.

FALCONER, D. S. 1964. Introduction to Quantitative Genetics. Oliver and Boyd, Edinburgh and London.

FERRIS, S. D., R. D. SAGE, E. M. PRAGER, U. RITTE AND A. C. WILSON. 1983a. Mitochondrial DNA evolution in mice. Genetics 105(3):681-721.

_____, _____, C.-M. HUANG, J. T. NIELSEN, U. RITTE AND A. WILSON. 1983b. Flow of mitochondrial DNA across a species boundary. Proc. Natl. Acad. Sci. USA 80:2290-2294.

GEIST, V. 1981. Behavior: adaptive strategies in mule deer. Pages 157-223 in O. C. Wallmo, editor. Mule and black-tailed deer of North America. Univ. Nebraska Press, Lincoln.

_____, AND P. KARSTEN. 1977. The wood bison in relation to hypotheses on the origin of the American bison. Z. Säugetierk. 42:119-127.

GYLLENSTEN, U., N. RYMAN, C. REUTERWALL AND P. DRATCH. 1983. Genetic differentiation in four European subspecies of red deer (Cervus elaphus). Heredity 51(3):561-580.

HARRIS, H. 1966. Enzyme polymorphisms in man. Proc. Roy. Soc. Ser. B. 164:298-310.

HARRISON, R., D. RAND AND W. WHEELER. 1985. Mitochondrial DNA size variation within individual crickets. Science 228:1446-1447.

HAUSWIRTH, W. AND P. LAIPIS. 1982. Mitochondrial DNA polymorphism in a maternal lineage of Holstein cows. Proc. Natl. Acad. Sci. USA 79:4686-4690.

HERRERO, S. 1972. Aspects of evolution and adaptation in American black bears and brown and grizzly bears of North America. Pages 221-231 in S. Herrero, editor. Bears: Their biology and management. Proc. of the international conf. on bear research and management, Calgary. IUCN Publ. New Ser. 23.

KRAMER, A. 1973. Interspecific behavior and dispersion of two sympatric deer species. J. Wildl. Manage. 37(3):228-300.

KURTEN, B. 1968. Pleistocene mammals of Europe. Aldine, Chicago.

_____. 1972. The age of mammals. Columbia Univ. Press, New York.

_____, AND E. ANDERSON. 1980. Pleistocene mammals of North America. Columbia University Press, New York.

LAIPIS, P., W. HAUSWIRTH, T. O'BRIEN AND G. MICHAELS. 1979. A physical map of bovine mitochondrial DNA from single animal. Biochimica et Biophysica Acta 565:22-32.

LANSMAN, R. A., R. O. SHADE, J. F. SHAPIRA AND J. C. AVISE. 1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. III. Techniques and potential applications. J. Mol. Evol. 17:214-226.

LEWONTIN, R. C. 1974. The genetic basis of evolutionary change. Columbia Univ. Press, New York and London.

_____, AND J. L. HUBBY. 1966. A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of Drosophila pseudoobscura. Genetics 54:595-609.

MACKIE, R. J. 1981. Interspecific relationships. Pages 487-507 in O. C. Wallmo, editor. Mule and black-tailed deer of North America. Univ. Nebraska Press, Lincoln.

MANIATIS, T., E. F. FRITSCH AND J. SAMBROOK. 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Habor, N.Y.

MAYR, E. 1970. Population, species and evolution. Harvard, Belknap Press, Cambridge, Mass.

McCLYMONT, R. A., M. FENTON AND J. R. THOMPSON. 1982. Identification of cervid tissues and hybridization by serum albumin. J. Wild. Manage. 46(2):540-544.

McDONALD, J. 1981. North American bison. U. of California Press, Berkeley, Los Angeles, London.

NEI, M. AND W.-H. LI. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76:5269-5273.

OSBORN, H. 1910. The age of mammals. MacMillan, New. York.

PEDEN, D. AND G. KRAAY. 1979. Comparison of blood characters in plains bison, wood bison and their hybrids. Can. J. Zool. 57:1778-1784.

POWELL, J. R. 1983. Interspecific cytoplasmic gene flow in the absence of nuclear gene flow: Evidence from Drosophila. Proc. Natl. Acad. Sci. USA 80:492-495.

_____, AND M. C. ZUÑIGA. 1983. A simplified procedure for studying mtDNA polymorphisms. Biochemical Genetics 21:1051-1055.

ROMER, A. 1966. Vertebrate paleontology. U. of Chicago Press, Chicago and London.

SCOTT, W. 1937. A history of land mammals in the western hemisphere. Hafner, New York.

SIBLEY, C. AND J. AHLQUIST. 1983. Phylogeny and classification of birds based on the data of DNA-DNA hybridization. Pages 245-292 in Current Ornithology Vol 1. Plenum, New York.

_____, _____. 1984. The phylogeny of the hominoid primates, as indicated by DNA-DNA hybridization. J. Mol. Evol. 20:2-15.

SMITH, M. H., R. BACCUS, H. O. HILLESTAD AND M. N. MANLOVE. 1984. Population genetics. Pages 119-128 in L. K. Halls editor. White-tailed deer, ecology and management. Stackpole books, Harrisburg, PA.

SPOLSKY, C., AND T. UZZELL. 1986. Evolutionary history of the hybridogenetic hybrid frog Rana esculenta as deduced from mtDNA analysis. Molec. Biol. and Evol. 3(1):44-56.

STORMONT, C., W. MILLER AND Y. SUZUKI. 1961. Blood groups and the taxonomic status of American buffalo and domestic cattle. Evolution 15:196-208.

TEMPLETON, A. 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution 37:221-244.

UPHOLT, W. B., 1977. Estimation of DNA sequence divergence from comparison of restriction endonuclease digests. Nucleic Acids Res. 4(5):1257-1265.

_____, AND I. DAWID. 1977. Mapping of mitochondrial DNA of individual sheep and goats: Rapid evolution in the D loop region. Cell 11:571-583.

WALLMO, O. C. 1981. Mule and black-tailed deer distribution and habitats. Pages 1-25 in O. C. Wallmo, editor. Mule and black-tailed deer of North America. Univ. Nebraska Press, Lincoln.

WATANABE, T., Y. HAYASHI, N. OGASAWARA AND T. TOMOITA. 1985. Polymorphism of mitochondrial DNA in pigs based on restriction endonuclease cleavage patterns. Biochemical Genetics 23:105-113.

WISHART, W. D. 1980. Hybrids of white-tailed and mule deer in Alberta. J. Mamm. 61(4):716-720.

WOLFE, J. R. 1983. Electrophoretic differentiation between Alaskan brown and black bears. J. Wildl. Manage. 47(1):268-271.

WRIGHT, J. W., C. SPOLSKY AND W. M. BROWN. 1983. The origin of the parthenogenic lizard Cnemidophorus laredoensis inferred from mitochondrial DNA analysis. Herpetologica 39(4):410-416.

YING, K., AND D. PEDEN. 1977. Chromosomal homology of wood bison and plains bison Can J. Zool. 55:1759-1762.

APPENDIX

Standard procedures for extraction of mtDNA from animal tissues adapted from Powell and Zuñiga (1984) and Davis et al. (1980) are given below. Keeping tissues and tubes on ice at all times unless otherwise noted:

1. Mince 10-25 g tissue from each animal with a scalpel. An electric blender at low speed may be useful for muscle samples.
2. Hand homogenize in an equal volume of homogenizing buffer (0.2 M sucrose, 1 mM EDTA, 2.5 mM $CaCl_2$, 0.03 M tris-HCl, 0.015% (v/v) Triton-X 100, final pH 7.5) in a dounce homogenizer until tissue is liquified.
3. Centrifuge 1000 X g for ten min. at 4C twice for brain or muscle, three times for liver or kidney. This removes nuclei and cellular debris.
4. Centrifuge the supernatant from step 3 at 14,000 X g 40 min at 4C to pellet mitochondria.
5. Suspend pellet in about 5 ml 0.25 M sucrose, 0.05 M $MgCl_2$ for a total volume of ten ml.
6. Add 1 ml DNase I (10 mg/ml stock) per 10 ml solution and icubate 30 min. at 37C. This removes nuclear DNA and is especially important when nuclear lysis is extensive as in tissues which have been frozen or exposed to warm temperatures post-mortem.

7. Add 1 ml 0.5 M EDTA per 10 ml solution to quench the DNase reaction.
8. Centrifuge at 14,000 X g 20 min at 4C to repellet mitochondria.
9. Suspend pellet in 5-10 ml 1 M EDTA, 0.2 M Tris-HCl pH 8.5
10. Add 1 ml 10% SDS (sodium dodecyl sulfate) per 10 ml solution to lyse mitochondria.
11. Add 100 ul proteinase-K (10mg/ml stock) per 10 ml solution and incubate at 60C for two hours.
12. Add 4 ml 5 M potassium acetate per 10 ml solution to precipitate SDS and proteins and leave on ice at least 30 min.
13. Centrifuge at least 9000 X g 45 min. at 4C.
14. Carefully remove clear supernatant containing nucleic acids without disturbing the SDS-protein pellet.
15. Precipitate nucleic acids with the addition of 2-3 times the solution's volume of 95% ethanol and keep at -20C 30-90 min. maximum.
16. Centrifuge at least 9000 X g for 30-60 min. at 4C to pellet nucleic acids.
17. Wash pellet in 70% ethanol to remove salts, and recentrifuge as in step 16 for 20 min.
18. Discard ethanol being careful not to pour out the pellet and allow the pellet to air dry.
19. Suspend pellet in 100-200 ul 0.01 M Tris, 5 mM EDTA, ph 7.8.

The final solution will be clear for brain and muscle and amber for liver or kidney. This yields about 2-5 ug mtDNA. Brain preparations are almost always digestable with restriction enzymes. Other tissues are usually digestable if processed within a month of death but may require use of excess enzyme during digestion or furthur purification by phenol extraction or cesium chloride centrifugation (Maniatis et al., 1982; Lansman et al., 1981).