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Forensic Identification of Ungulate Species Using Restriction Digests of PCR-Amplified Mitochondrial DNA

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ABSTRACT: A survey of mitochondrial D-loop variation in 15 species of ungulates was conducted via amplification by the polymerase chain reaction followed by restriction fragment length polymorphism analysis. This survey included moose (*Alces alces*), caribou (*Rangifer tarandus*), mule deer (*Odocoileus hemionus hemionus*), black-tailed deer (*O. h. columbianus*), white-tailed deer (*O. virginianus*), wapiti (*Cervus elaphus*), pronghorn antelope (*Antilocapra americana*), bighorn sheep (*Ovis canadensis*), Stone's sheep (*O. dalli*), domestic sheep (*O. aries*), moulflon sheep (*O. musimon*), mountain goat (*Oreamnos americanus*), domestic goat (*Capra hircus*), domestic cattle (*Bos taurus*), and bison (*Bison bison*). The results of this preliminary survey indicate that there may be sufficient species specific variation in the D-loop region of the mitochondrial genome of the ungulate species examined here, with the exception of deer (*Odocoileus*) species, to establish the species origin of the mitochondrial haplotypes of this group. The Odocoileus species are known to hybridize and sharing of mtDNA haplotypes was observed. The chelex DNA extraction technique was successfully used on small blood stains.

KEYWORDS: forensic science, mitochondrial DNA, PCR, species identification, ungulates

The development of molecular techniques such as restriction fragment analysis (i.e., RFLPs), DNA fingerprinting [*1*] and most recently the use of the polymerase chain reaction (PCR) in the amplification of individual loci, for example, the HLA DQα locus [*2*] and microsatellites [*3,4*] have revolutionized the field of human forensic science. With these techniques, the molecules responsible for heredity, DNA, can be used directly to describe and determine the amount of genetic difference between species, subspecies, populations, and individuals. In wildlife forensic investigations these procedures are just beginning to be used [*5–10*].

One application of DNA analysis to wildlife forensics is the identification of species origin of biological samples. For example, it could be used to determine if a meat product being sold for domestic consumption is derived from a domestic animal, a particular species of wildlife, or both. Further, it could be used to identify the species origin of blood stains and small forensic samples. In the past, these questions were being addressed through the use of protein electrophoretic and immunological techniques [*11–15*]. The latter have been generally successful, however, they do possess some inherent weaknesses. For instance, tissue samples may be too small to analyze or uncharacterizable due to protein denaturation caused by cooking or tissue decomposition. Recently a number of DNA based species identification procedures have been developed [*7–10*]. Some of these techniques require high quality or modest quantities of DNA [*7,16,19*]. The use of the polymerase chain reaction (PCR) [*8,9*] to amplify a specific region of DNA from degraded and/or small quantities of forensic sample overcomes these obstacles. PCR has been used to analyze DNA from degraded samples such as ancient samples [*17,18*] and extremely small samples [*19*], including blood stains [*20,21*].

The choice of a genetic marker is directly related to the question being asked. For species identification the marker should display enough variation among species so that each species has a unique set of variants yet homogeneous enough within a species so that a variant can be correctly assigned to a species. Mitochondrial DNA (mtDNA) is known to possess these characteristics. Its usefulness in wildlife forensics has been noted [for example, *7,8*], and it has been used in a large number of studies of wildlife populations. The D-loop region of mtDNA has a fast rate of base substitution, about 4.7×10^{-8} per site per year [*22*], and therefore should display a large amount of sequence divergence among different species. The variation in this area should be detectable by the use of four base recognition restriction enzymes. The use of restriction enzymes to characterize a genetic marker has some advantages over DNA sequencing. First, it is less expensive and time consuming. Second, it allows samples containing more than one species to be easily determined while a DNA sequence derived from such a sample may be unreadable due to overlapping sequences.

The objective of this study is to examine the usefulness of such a marker system in the identification of ungulate species. Reported here are the findings of the restriction analysis of the D-loop of 15 species of ungulates. The results of this preliminary survey indicate that there seems to be sufficient species specific variation in the D-loop region of the mitochondrial genome of the ungulate species examined here, with the exception of deer species, to establish the species origin of the mitochondrial haplotypes of this group. In 8 of 10 test samples the species origin(s) of amplified DNA from either steaks, hamburger or sausages has been determined. In the remaining two cases the species origin has been tentatively classified. Additionally, the use of the chelex extraction technique to extract DNA of sufficient quality for amplification from deer blood stains has been demonstrated.

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TABLE 1—*Sample type, collection location, and haplotype of samples in forensic study. Letters in brackets are the species code used throughout the text in discussion of haplotypes.*

Species	Sample #	Collection location or type	Haplotype
Moose			
Alces alces (A.a.)	4	Jasper NP, AB	A.a.-A
	2	Banff NP, AB	A.a.-A
	2	Terra Nova NP, NFD	A.a.-A
Caribou			
Rangifer tarandus (R.t.)	1	Delta herd, Alaska	R.t.-A
	1	Barren Ground Southampton Is., NWT	R.t.-A
	1	Woodland, Jasper NP, AB	R.t.-A
	1	George's R. herd, NFD	R.t.-B
	1	Porcupine herd, Alaska	R.t.-C
	1	Peary, Queen Elisabeth Is., NWT	R.t.-C
	1	European Reindeer, Alaska	R.t.-C
Mule deer			
Odocoileus hemionus hemionus (O.h.h.)	1	Banff NP, AB	O.h.h.-A
	1	Murray Lake, AB	O.h.h.-A
	1	Wainwright, AB	O.h.h.-A
	1	Swan Hills, AB	O.h.h.-A
	1	Cavendish, AB	O.h.h.-A
	1	Vetchland, AB	O.h.h.-A
	1	Saskatchewan	O.h.h.-A
	1	Vernon, BC	O.h.h.-B
	1	Banff NP, AB	O.h.h.-C
	1	Drayton Valley, AB	O.h.h.-D
	1	Banff NP, AB	O.h.h.-E
	1	Wainwright, AB	O.h.h.-E
Black-tailed deer			
O.h. columbianus (O.h.c.)	1	Pemberton, BC	O.h.c.-A
	1	Texada Is., BC	O.h.c.-A
	1	Port Renfrew, Van. Is., BC	O.h.c.-A
	1	Salmon R., Van. Is., BC	O.h.c.-A
	1	Salmon R., Van. Is., BC	O.h.c.-B
White-tailed deer			
O. virginianus (O.v.)	1	Banff NP, AB	O.v.-A
	1	Rocky Mt. House, AB	O.v.-A
	1	Edson, AB	O.v.-A
	1	Robinson, AB	O.v.-A
	1	Lesser Slave Lk., AB	O.v.-A
	1	Mannville, AB	O.v.-A
	1	Debolt, AB	O.v.-A
	1	Wainwright, AB	O.v.-A
	1	Manitoba	O.v.-A
	1	Saskatchewan	O.v.-B
	1	Banff NP, AB	O.v.-C
	1	Kootney NP, BC	O.v.-C
Wapiti			
Cervus elaphus (C.e.)	4	Vancouver Is., BC	C.e.-A
	1	Benson R., Van. Is., BC	C.e.-A
	1	Campbell Lk., Van. Is., BC	C.e.-A
	1	Gold R., Van. Is., BC	C.e.-A
	1	Frog Lk., Van. Is., BC	C.e.-A
	1	Compton Cr., Van. Is., BC	C.e.-A
	1	Rocky Mt. House, AB	C.e.-C
	1	Jasper NP, AB	C.e.-C
	1	Carrot Cr., AB	C.e.-C
	1	Edson, AB	C.e.-C
Pronghorn antelope			
Antilocapra americana (An.am.)	1	Milk River, AB	An.am.-A
	1	Wardlow, AB	An.am.-B
	2	Brooks, AB	An.am.-A
	1		An.am.-A
	1	Valley Zoo, Edmonton, AB	An.am.-A
Bighorn & Stone's sheep			
Ovis canadensis (Ov.c.)	1	Willmore, AB	Ov.c.-A
	1	Blairmore, AB	Ov.c.-A
	1	Kootney NP, BC	Ov.c.-A
	1	Grand Cache, AB	Ov.c.-A
	2	Banff NP, AB	Ov.c.-A
	1	Jasper NP, AB	Ov.c.-A
Ovis dalli (Ov.d.)	1	Yukon T.	Ov.d.-A
Domestic & Mouflon sheep			
Ovis aries (Ov.ar.)	1	Finn sheep	Ov.ar.-A
	1	Romanov sheep	Ov.ar.-A
	1	Merrino sheep	Ov.ar.-A
	2	Suffolk sheep	Ov.ar.-B
	1		Ov.ar.-B
Ovis musimon (Ov.m.)	1	Mouflon sheep	Ov.m.-A = Ov.ar.-B
Mountain goat			
Oreamnos americanus (Or.am.)	1	Caw Ridge, AB	Or.am.-A
	1	Grande Prairie, AB	Or.am.-A
	1	Mt. Hammel, AB	Or.am.-A
	1	Canmore, AB	Or.am.-A
	1	Banff NP, AB	Or.am.-B
	1	Jasper NP, AB	Or.am.-B
	1	Meager Mt., BC	Or.am.-C
	1	Ryan Creek, BC	Or.am.-D
Domestic goat			
Capra hircus (Ca.h.)	2	Alpine goat	Ca.h.-A
	4	Nubian goat	Ca.h.-A
	1	Nigerian Dwarf goat	Ca.h.-A
	1	Angora goat	Ca.h.-A
Domestic cattle			
Bos taurus (B.t.)	1	Charolais	B.t.-A
	1	Charolais X	B.t.-A
	1		B.t.-A
	1	Simmental/Angus	B.t.-B
	1	Hereford X	B.t.-B

Materials and Methods

D-loop Restriction Digest Survey

Sample Collection—Preliminary sample sets (Table 1) were established from existing tissue collections; Alberta Fish and Wildlife and Parks Canada. Most of these samples were collected in the provinces of Alberta (AB) and British Columbia (BC). For each species, samples were chosen to represent as wide a geographic range as possible. Moose (*Alces alces*) populations are found throughout Canada and the northern United States. The samples analyzed are from the 2 subspecies *A. a. andersoni* (samples from the Canadian Rocky Mountains) and *A. a. americana* (samples from Newfoundland). *A. a. andersoni* is found in western Canada and the Northwest Territories and *A. a. americana* is found in eastern Canada and the northeastern United States. Caribou (*Rangifer tarandus*) are also widespread in the northern half of North America. Individuals from seven distinct herds representing all 5 subspecies (*R. t. tarandus, R. t. caribou, R. t. groenlandicus, R. t. pearyi,* and *R. t. granti*) found in North America are used in this study. Mule deer (*Odocoileus hemionus*) is a western North American species extending from southern Yukon to Mexico. The two subspecies examined are dealt with separately. *O. h. hemionus* samples are from the northern third of the subspecies range; western Canada (primarily AB). Black-tailed deer samples (*O. h. columbianus*) were also collected from the northern third of this coastal

subspecies range, southwestern BC. White-tailed deer (*O. virginianus*) occupies areas of southern Canada, the lower United States, excluding the southwestern States, and extends through Mexico and Central America into South America. Two subspecies were examined; a northwestern United States subspecies (*O. v. ochrourus*) which extends into southern BC and a prairie subspecies (*O. v. dacotensis*) found in Wyoming, Montana, the Dakotas, and the Prairie Provinces. The sample of *O. v. ochrourus* are from the Canadian Rocky Mountains and the samples of *O. v. dacotensis* are from the Canadian Prairie Provinces (primarily AB). Remnants of wapiti (*Cervus elaphus*) populations are found throughout North America. The samples examined are from the western subspecies (*C. e. nelsoni*), collected in the Canadian Rocky Mountains, and a west coast subspecies (*C. e. roosevelti*), collected from Vancouver island. The range of the pronghorn antelope (*Antilocapra americana*) is situated in western North America extending from the southern areas of the Prairie Provinces to northern Mexico. Animals from the northern limit of the range, in southern AB, were examined. Bighorn sheep (*Ovis canadensis*) is another western species occurring in the Cordilleran region of western North America from the Canadian Rockies to northern Mexico. The samples examined are from throughout the Canadian Rockies, a large part of the range of the subspecies *O. c. canadensis*. A single sample of Stone's sheep (*O. dalli stonei*), a northern species found in the Yukon and Alaska, was also included in the analysis. Mountain goat (*Oreamnos americanus*) was the last wild species examined in this survey. Mountain goats are confined to the Cordilleran region of Northwest America, primarily in BC but extending into southeastern Alaska and northern Montana. The samples collected in AB are from the Canadian Rocky Mountains while the BC are from the Coastal Range. Species distributions were taken from Banfield [*23*] and Hall [*24*].

Isolation of DNA—Total DNA was isolated from 0.5 g of muscle from each of the 99 samples representing 14 species of ungulates listed on Table 1. Tissue was frozen in liquid nitrogen, ground using a mortar and pestle and then resuspended in 1.0 mL of phosphate buffered saline. DNA was extracted using the Applied Bioscience DNA extractor, model 341, which lysed the cells and then removed the protein and other cellular debris through phenol/chloroform extractions. All purified DNA was dissolved in TE (10 mM Tris, 1 mM EDTA) and stored at −70°C until it was analyzed.

In addition to those individuals examined in this study results of previous research on D-loop variation were also available. A survey of D-loop variation in North American elk populations has found only one other haplotype besides the two characterized here [*23*]. This haplotype has been included in the results and is labelled C.e.-B (see Table 2). Complete D-loop sequence variation of domestic cattle [*26*] and 29 North American bison [*27*] is also available for comparison and the inferred restriction digests of these samples have been included in Table 2.

PCR Amplification—Slightly modified versions of the 'universal' D-loop primers H00651 and L15926 [*28*] were used successfully to amplify the D-loop in each of the 15 species examined. These primers are

5′ to 3′ GGGTCGGAAGGCTGGGACCAAACC and

5′ to 3′ TAATATACTGGTCTTGTAAACC respectively.

The thermal cycling was conducted on a Perkin-Elmer-Cetus model 480. Ten to 100 ng of total DNA was used as a template for the reaction conducted in a 100 μL volume. The reagent concentrations were 2.0 mM $MgCl_2$, 200 nM dNTP's, 10 μL of 10 reaction buffer (Promega), 20 pmol of each primer and 1 unit of Taq DNA polymerase (Promega). The typical thermal cycling required for the enzymatic reaction involved an initial cycle of 94°C for 5 minutes, 52°C for 30 s and 72°C for 2 minutes, 30 cycles of 94°C for 15 s, 52°C for 30 s and 72°C for 2 minutes and a final cycle of 94°C for 15 s, 52°C for 30 s and 72°C for 10 minutes.

The optimal annealing temperature for the enzymatic amplification of the mitochondrial D-loop was slightly different for each species examined. This is the temperature at which the D-loop product is maximized and the secondary products are minimized. The optimum annealing temperatures are 52°C for domestic sheep, cattle, bison and mountain goats, 53°C for domestic goats, 54°C for caribou, bighorn and Stone's sheep, pronghorn, wapiti and white-tailed deer and 56°C for mule deer, black-tailed deer and moose.

If production of secondary product was a problem in characterization of D-loop product an additional step of isolation of D-loop product was required. Amplification products were separated by electrophoresis on a low melting point (LMP) agarose gel in a Tris-acetate buffer. The D-loop band was excised from the gel, added to 10 volumes of H_2O and heated for 10 minutes at 65°C. The resulting solution containing only the D-loop product and was then used as the template in subsequent PCR amplifications used to characterize the molecule.

Restriction Digestion Analysis—The six, four base pair recognition, restriction enzymes, Alu I, Cfo I, Hae III, Hinf I, Hpa II and Rsa I were used to characterize the D-loop products. For each enzyme 15 μL of PCR product was digested with 3 units of enzyme at 37°C for at least 2 hours. The resulting restriction fragments were visualized by electrophoresis through a 4% and/or 5% acrylamide gel, which was then stained with ethidium bromide and exposed to UV light. The base pair sizes of the fragments were estimated to 5 bp intervals by comparison to a 123 bp ladder (BRL).

Amplification of Small Quantities of DNA

Chelex Extraction—DNA was extracted from 3 mm^2 blood stains on brown paper, 5 mm blood stained cotton strands and 3 μL of whole blood by a chelex extraction technique [*20,21*] from a mule deer and a white-tailed deer sample. In each case the blood sample was placed in 200 μL of a 5% chelex solution (pH-10) and incubated at 56°C for 30 minutes. The solution was then vortexed at high speed for 10 s, incubated at 100°C for 8 minutes, vortexed again for 10 s and centrifuged for 3 minutes.

PCR Amplification of Chelex Extracted DNA—Twenty μL of the above chelex extract was used as a template for the enzymatic amplification. This reaction was contained in a 50 μL volume and overlaid with 50 μL of heavy mineral oil. The reagent concentrations were 3.0 mM $MgCl_2$, 200 nM dNTP's, 5 μL of 10X reaction buffer (Promega), 10 pmol of each primer and 2 units of Taq DNA polymerase (Promega). The thermal cycling of the reaction was similar to that described except the annealing temperature was 54°C and the middle cycle was repeated 40 times.

Test Cases

DNA was isolated from 10 tissue samples including steaks, hamburger and sausages of unknown species origin. This DNA

TABLE 2—*Results of the D-loop restriction enzyme survey. Size of fragments is given in base pairs. The maximum estimate of D-loop size is listed for each haplotype.*

Species	Enzyme	Haplotype	Sizes
Moose	Alu I	A	390 250 175 90 85 80
(*A. alces*)	Cfo I	A	975 130
	Hae III	A	585 365 150
$A_{max.}$-1175	Hinf I	A	725 395
	Hpa II	A	575 365 235
	Rsa I	A	860 160 50 45 40
Caribou	Alu I	A	350 280 175 90 85 80
(*R. tarandus*)		B,C	350 275 175 90 85 80
	Cfo I	A,B,C	990 135
$A_{max.}$-1275	Hae III	A	600 450 150
$B_{max.}$-1250		B	585 430 150
$C_{max.}$-1225		C	585 420 150
	Hinf I	A	745 475
		B	425 410 185 125
		C	745 440
	Hpa II	A	595 445 235
		B	595 425 230
		C	575 415 230
	Rsa I	A,C	945 265
		B	675 265 125 60
Mule deer	Alu I	A,D	410 240 175 95 90 85 80
(*O. h. hemionus*)		B	410 310 175 95 90 85
		C	420 310 175 95 90 85
$A,B,C,D_{max.}$-1365		E	435 195 175 120 95 90 85
$E_{max.}$-1460	Cfo I	A,B,C,D	1230 135
		E	1325 135
	Hae III	A,B,D	635 465 150
		C	640 465 150
		E	670 475 150
	Hinf I	A,B,C,D	770 300 180
		E	880 300 180
	Hpa II	A,B	635 445 235
		C	660 445 235
		D	635 265 235 180
		E	685 265 235 180
	Rsa I	A,B,C,D	705 250 150 45 40
		E	705 250 150 75 45 40
White-tailed deer	Alu I	A,C	420 310 175 95 90 85
(*O. virginianus*)		B	410 240 175 95 90 85 80
	Cfo I	A,B,C	1230 135
$A,B,C_{max.}$-1365	Hae III	A,C	640 465 150
		B	635 465 150
	Hinf I	A,B,C	770 300 180
	Hpa II	A,C	660 445 235
		B	635 445 235
	Rsa I	A,B	705 250 150 45 40
		C	705 300 150 45 40
Black-tailed deer	Alu I	A,B	445 315 175 95 90 85
(*O. h. columbianus*)	Cfo I	A,B	1285 130
	Hae III	A,B	650 465 150
$A,B_{max.}$-1415	Hinf I	A	820 475
		B	820 450
	Hpa II	A	675 255 235 180
		B	675 425 255
	Rsa I	A,B	995 305 40
Wapiti	Alu I	A,B,C	340 275 205 110 95 90 80
(*C. elaphus*)	Cfo I	A,C	660 375 280
		B	440 375 280 220
$A,B,C,_{max.}$-1315	Hae III	A,B,C	615 440 140
	Hinf I	A,B	450 340 330 135
		C	750 340 135
	Hpa II	A,B,C	615 445 230
	Rsa I	A,B,C	905 280 50 45
Pronghorn	Alu I	A,B	360 290 210 95 90 85
(*A. americana*)	Cfo I	A,B	695 385 80
	Hae III	A,B	520 400 145
$A,B_{max.}$-1165	Hinf I	A	685 430
		B	565 430 125
	Hpa II	A,B	615 515
	Rsa I	A,B	705 220 55 50 45 40
Bighorn & Stone's Sheep (*O. canadensis* and *O. dalli*)	Alu I	A	360 260 135 90 75
	Cfo I	A	1325
	Hae III	A	650 380 145
	Hpa II	A	660 375 235
$A_{max.}$-1325 (both)	Rsa I	A	460 290 250 75 45 40
-*O. canadensis*	Hinf I	A	620 360 125
-*O. dalli*	Hinf I	A	440 355 175 125
Domestic sheep	Alu I	A,B	655 255 90 75
(*O. aries*)	Cfo I	A,B	1525
	Hae III	A,B	835 380 145
$A,B_{max.}$-1525	Hinf I	A,B	880 345
	Hpa II	A,B	815 365 230
	Rsa I	A	460 320 250 60 50 45 40
		B	740 320 80 45 40
Mountain goats	Alu I	A,B,C,D	360 250 210 175 90 85 80
(*O. americanus*)	Cfo I	A,C,D	745 710
		B	1695
$A_{max.}$-1480	Hae III	A,B,C,D	915 365 145
$B_{max.}$-1695	Hinf I	A,B,C,D	1005 385
$C,D_{max.}$-1495	Hpa II	A,B	885 350 245
		C,D	740 355 245 155
	Rsa I	A,B,C	775 380 60 55 45 40
		D	775 365 50 45 40
Domestic goats	Alu I	A	710 260 175 95 90 85
(*C. hircus*)	Cfo I	A,B	1605
	Hae III	A	890 390 145
$A_{max.}$-1605	Hinf I	A	705 405 130
	Hpa II	A	885 375 245
	Rsa I	A	775 345 105 60 55
Bison[a]	Alu I	A,B	267 245 230 144 94 92 41
(*B. bison*)	Cfo I	A,B	1112
	Hae III	A,B	530 248 145 116 63 10
A-1112	Hinf I	A	676 328 58 51
	Hinf I	B	556 328 120 58 51
	Hpa II	A,B	527 356 230
	Rsa I	A,B	710 206 116 35 20 19 7
Domestic cattle	Alu I	A[a],B	266 250 246 144 94 92 41
(*B. taurus*)	Cfo I	A[a],B	1132
	Hae III	A[a],B	550 249 145 116 63 10[a]
A,B-1132	Hinf I	A[a],B	695 329 58 51
	Hpa II	A[a]	546 357 230
		B	546 240 230 115
	Rsa I	A[a],B	711 295 40 35 26[a] 19[a] 7[a]

[a]These fragment sizes were inferred from previously known D-loop sequences.

was then used as a template for an initial PCR reaction. The PCR reaction cycles were similar to that listed in the D-loop survey except an annealing temperature of 52°C was used. The reaction was contained within a 50 μL volume and the reagent concentrations were 2.0 mM $MgCl_2$, 200 nM dNTP's, 5 μL of 10 reaction buffer (Promega), 2 units Taq DNA polymerase (Promega) and 10 pmol of each primer. If more than one product resulted from the initial PCR the products were separated on a 2% LMP gel, isolated from each other, diluted 10 with H_2O and used as a template for a secondary PCR. The single products resulting from the secondary PCR were then digested with the restriction enzymes Cfo I, Alu I and Rsa I and electrophoresed on either a 4% or 5% acrylamide gel with digests of known species' PCR products.

Results

D-loop Restriction Digest Survey

The results of the survey for each of the six enzymes are summarized in Table 2. Included in this Table are the restriction digest

fragments which were calculated from the known D-loop sequence of 1 cow [26] and 31 North American bison [27]. Table 2 will serve as a data base for species identification. In total, 31 haplotypes were observed. These haplotypes are characterized by either restriction enzyme site differences or, more commonly, size differences between the resulting DNA fragments produced by homologous restriction sites.

In the following sections the variation listed in Table 2 will be described in regards to both intraspecific and interspecific variation although the unique aspects of the interspecific variation will be stressed. Haplotype names are shortened to a unique code to conserve space (see Table 1).

Moose—A single haplotype was found in all 8 moose samples (Table 1). This haplotype *Alces alces* (A.a.)-A is easily characterized by its small overall size which is illustrated by the relatively small fragment sizes observed in the Cfo I and Hae III digests (Table 2, Fig. 1*a*). The restriction digestion fragment patterns are similar to deer and may indicate A.a.-A has no unique restriction site for the enzymes surveyed.

Caribou—The 7 caribou samples, representing five subspecies (Table 1), were collected from widespread geographic locations. The two main haplotypes *Rangifer tarandus* (R.t.)-A and R.t.-C appear to have similar restriction sites and differ only in fragment sizes. The third haplotype R.t.-B is different from the other two haplotypes by 2 Hinf I and 1 Rsa I restriction sites (Table 2, Fig. 1*c*). This makes the R.t-B allele easily distinguishable from all other alleles in this survey. The more common R.t-A and C alleles have similar restriction patterns to those of deer and moose, differing mainly in fragment size. The Alu I digest (Fig. 1*b*) displays identifiable restriction size differences.

Mule Deer—Five mitochondrial haplotypes were observed in the 12 individuals sampled (Table 1). The most common haplotype, mule deer *Odocoileus hemionus hemionus* (O.h.h.)-A, is present in seven of the individuals. This haplotype can be identified by Alu I and Hpa II digests. The two haplotypes O.h.h.-B and O.h.h.-C, each seen in one individual, are identical in restriction sites to the most common white-tailed deer haplotype *O. virginianus* (O.v.)-A (Table 2), but differ in some fragment sizes. The last two haplotypes O.h.h.-D and O.h.h.-E both possess unique Hpa II restriction sites. In addition, haplotype O.h.h.-E possesses an unique Alu I restriction pattern which can be explained by 2 restriction site changes from the O.h.h.-A Alu I digest (Fig. 1*b*).

White-Tailed Deer—Three haplotypes were detected in 12 individuals (Table 1). The most common haplotype (9 of 12), O.v.-A, as stated above is similar to the rare mule deer haplotypes O.h.h.-C and -B. Conversely the white-tailed O.v.-B haplotype, present in a single individual, is identical to the most common mule deer haplotype, O.h.h.-A. The last white-tailed deer haplotype O.v.-C differs from O.v.-A only in the Rsa I digest (Table 2, Fig. 1*c*). Both are distinguishable from all other mule deer haplotypes by their Alu I (Fig. 1*b*) and Hpa II digest (Table 2).

Black-Tailed Deer—In the five black-tailed deer samples 2 haplotypes were found; *O. h. columbianus* (O.h.c.)-A (4 of 5) and O.h.c.-B (Table 1). Both black-tailed deer haplotypes differ from other deer haplotypes in the loss of at least one restriction site each in the Rsa I (Fig. 1*c*) and Hinf I digestions.

Wapiti—Thirteen elk samples were included in this present survey in which two haplotypes *Cervus elaphus* (C.e.)-A (9 of 13) and C.e.-C (4 of 13) were found (Table 1). In this survey and in a separate more thorough survey of 49 individuals [23] haplotype C.e.-A was only found in Roosevelt elk (*C. e. roosevelti*) populations. All Roosevelt elk examined (9 and 16 respectively) possess this haplotype. Only one other haplotype, in the more extensive survey, was found and it is labelled C.e.-B. These elk haplotypes may be characterized from all other species presented here by their Cfo I and Hinf I digestions (Table 2, Fig. 1*a*). In most other cases its restriction fragment patterns are similar to moose, deer and caribou.

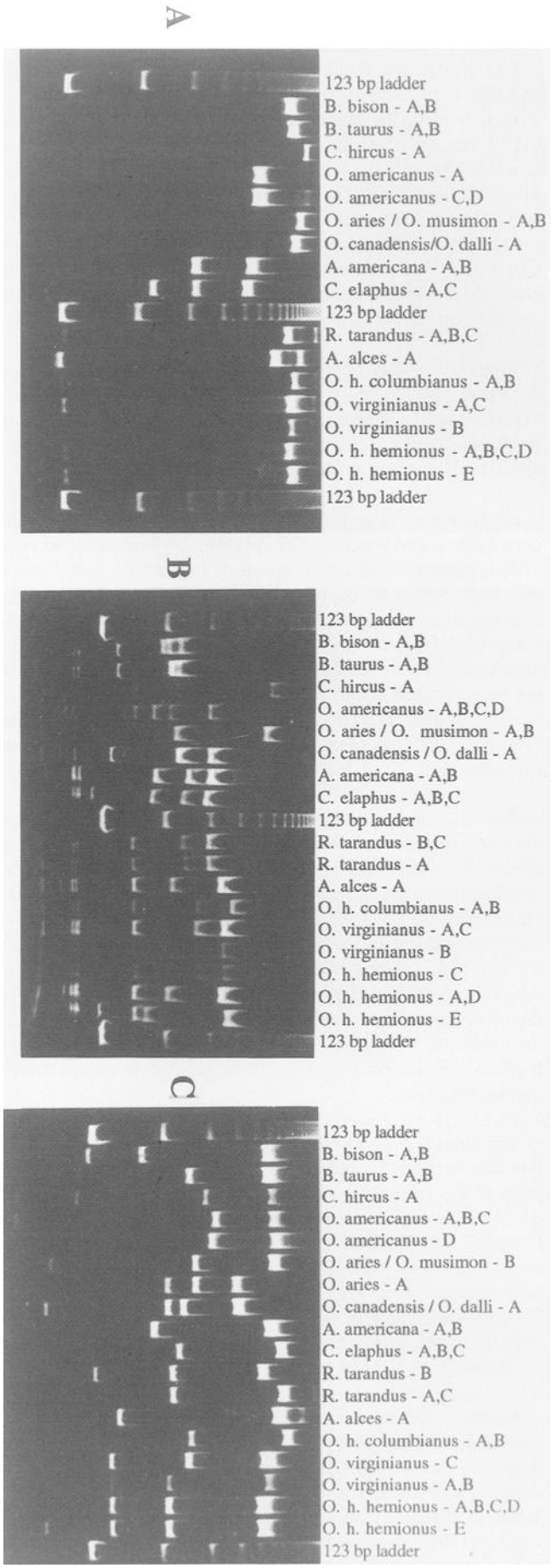
Pronghorn—Two haplotypes, *Antilocapra americana* (An.am.)-A (5 of 6) and An.am.-B (1 of 6), differing in the Hinf I digestion (Table 2) were detected in the 6 pronghorn samples examined. Both these haplotypes are characterized by unique Cfo I (Fig. 1*a*) and Hpa II restriction fragments (Table 2).

Bighorn and Stone's Sheep—A single haplotype, *Ovis canadensis* (Ov.c.)-A, was found in the seven bighorn sheep examined. This haplotype is part of a group of haplotypes found in sheep and goats which all contain a number of distinct restriction site changes from pronghorn and the Cervidae species. The Stone's sheep haplotype, *O. dalli* (Ov.d.)-A, is identical to Ov.c.-A in all digests except Hinf I (Table 2). Here the Stone's sheep haplotype possesses an additional restriction site. The Ov.c.-A and Ov.d.-A haplotypes differ from domestic sheep haplotypes in a large number of restriction fragment sizes and additional Alu I and Hinf I restriction sites (Fig. 1*b*, Table 2).

Domestic and Mouflon Sheep—Two haplotypes are present in the seven samples examined (Table 1). The first haplotype *O. aries* (Ov.ar.)-A (3 of 7) possesses similar digestion fragment patterns, but differs in size, to the wild sheep above in the Cfo I, Hae III, Hpa II and Rsa I digests (Fig. 1*a* and 1*c*, Table 2). Restriction site differences occur in the Alu I and Hinf I digests (Fig. 1*b*, Table 2). The Hinf I digest is similar to that found in mountain goats while the presence of a single Alu I restriction site is unique to domestic sheep. The second haplotype Ov.ar.-B is present in 3 of the domestic sheep and in the mouflon sheep examined. This haplotype is identical to Ov.ar.-A in all digests except the Rsa I digest (Fig. 1*c*).

Mountain Goat—In the eight mountain goat samples 4 haplotypes were characterized (Table 1). All mountain goat haplotypes possess the same unique Alu I digestion pattern (Fig. 1*b*, Table 2). Most haplotypes, *Oreamnos americanus* (Or.am.)-A (4 of 8), Or.am.-C and Or.am.-D, also contain a unique Cfo I restriction site which cleaves the D-loop molecule into two roughly equal fragments (Table 2, Fig. 1*a*). A fourth haplotype, Or.am.-B (2 of 8), does not contain this Cfo I restriction site, a character similar with sheep and domestic goat, but is otherwise identical to Or.am.-A (Table 2).

Domestic Goat—A single haplotype, *Capra hircus* (Ca.h.)-A was detected in all 7 samples examined (Table 1). This haplotype possesses similar restriction fragment patterns to other goats and sheep in the Cfo I, Hae III, Hinf I and Hpa II digests (Fig. 1*a*, Table 2). Unique restriction digest patterns were observed in the Alu I and Rsa I digests (Fig. 1*b* and 1*c*). The Alu I digest is similar to mountain goat except for loss of a restriction site. The Rsa I



digest of Ca.h.-A produces a 109-bp fragment not observed in any other goats or sheep.

Bison—Of the 31 North American bison sequenced [*27*] 2 haplotypes are characterized using the six restriction enzymes describe here. These haplotypes differ in only an additional Hinf I restriction site. Both these haplotypes are similar to sheep, goats and cattle in that there is no Cfo I restriction site present. They differ from goats and sheep in Alu I and Rsa I restriction sites and in the comparatively smaller size of the mitochondrial D-loop (1112-bp) (Fig. 1*b* and 1*c*, Table 2).

Domestic Cattle—Two cattle haplotypes were observed in a survey of 6 individuals including 5 samples analyzed here (Table 1) and one published sequence [*26*]. These haplotypes are identical in all restriction digests except Hpa II (Table 2), and are very similar to the bison haplotypes. The main differences between the haplotypes are the result of a 20-bp insert in the cattle haplotype (1132-bp) and two unique Rsa I restriction enzyme sites (one each, Fig. 1*c*).

Swine, Horse, and Donkey—The D-loop primers used here were also tested for their ability to amplify swine, horse and donkey D-loop products. In these cases the PCR amplification yielded nothing or weak multiple products consistent with poor primer affinity for these species.

Chelex Extraction and D-loop Amplification

It is important for forensics that species can be identified from small amounts of material. Therefore, DNA was extracted from 3 mm^2 blood stains on brown paper, 5 mm blood stained cotton strands and 3 μL of whole blood from separate mule deer and white-tailed deer samples by a chelex extraction technique. D-loop product from the chelex extract was amplified and then digested with Hpa II. The restriction fragment digests had the patterns of O.h.-A and O.v.-A haplotypes (results not shown).

Test Cases

To date ten case samples of unknown species origin have been examined utilizing this technique (see Table 3). In five samples the D-loop amplified was unambiguously typed and found to be of a single species origin. In two cases a new and unique haplotype was encountered. This haplotype was very similar to the deer haplotypes (similar in restriction sites and fragment sizes in all digests except Rsa I) and has been tentatively classified as of deer origin. In three cases two D-loop products were amplified from the meat product (i.e., sausage) under examination and therefore appear to be of dual sample origin. In each case the two PCR products were isolated and subsequently identified. Figure 2 shows one such case in which two D-loop products were observed. The D-loop products in this case were of deer and caribou origin.

FIG. 1—*Restriction digests of selected mitochondrial D-loop haplotypes of each wildlife species examined, electrophoresed on 5% acrylamide gels. For a complete listing of haplotypes and the approximate base pair sizes of the fragments see Table 2. Each sample is labelled with the corresponding haplotype listed in Table 1. Figure* a, b *and* c *represent Cfo I, Alu I and Rsa I digests respectively.*

TABLE 3—*Results of forensic test cases: Sample type and species origin.*

Case #	Sample type	Species origin
F1	Steak	Cattle
F2	Sausage	Wapiti
F3	Hamburger	Moose
F4	Steak	Deer[a]
F5a	Sausage	Deer
b	Sausage	Deer, cattle
c	Sausage	Deer, caribou
d	Sausage	Cattle
F6	Steak	Deer[a]
F7	Sausage	Deer, caribou

[a]Haplotype similar to deer.

Discussion

There seems to be sufficient species specific variation in the D-loop region of the mitochondrial genome of the ungulate species examined here to establish the species origin of the mitochondrial haplotypes of this group. Once a D-loop product has been amplified (and purified if necessary) species identification can be preliminary established through the use of the three restriction enzymes Cfo I, Alu I and Rsa I (see Fig. 3). First, Cfo I (Fig. 1*a*) is used to identify the four major groups in this study (1) Cervinae (elk) and (2) Odocoilinae (deer, moose and caribou) within the Cervidae and (3) Antilocaprinae (pronghorn) and (4) Bovinae/Caprinae within the Bovidae (cattle, bison, sheep and goats) [Classification as in *29*]. The restriction enzymes Alu I (Fig. 1*b*) and Rsa I (Fig. 1*c*) are then used to identify the species (Hinf I may also be used). In forensic cases, direct comparison of known and unknown sample Alu I and Rsa I digests would be desirable to establish a positive identification (for example, Fig. 2).

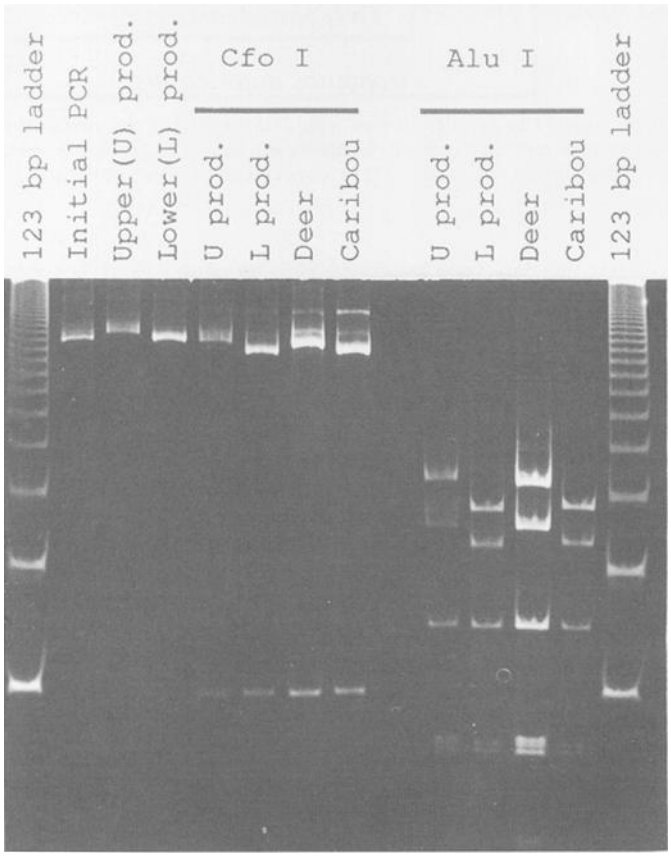


FIG. 2—*Example of the restriction digest of 2 D-loop haplotypes found within a sample. The two initial products were isolated and reamplified as described in the methods, and then digested with the restriction enzymes Alu I and Cfo I. D-loop restriction patterns of deer and caribou origin are present for comparison.*

As noted in the results section the origin of deer mitochondrial haplotypes can only be known to the genus level. The two deer species share mtDNA haplotypes indicating the retention of ancestral haplotypes or alternatively, as the species are known to hybridize, the introgression of mtDNA [*30,31*]. In either case, common white-tailed deer D-loop haplotypes are found at low frequencies in mule deer populations and visa versa as noted here. A similar situation occurs between mule and black-tailed deer. Because of the uncertainty this causes in deer species identification, this single marker system would not be practical in forensic case work involving deer. However, when additional nuclear genetic marker systems are developed this problem should be overcome. By characterizing both nuclear and mitochondrial genetic markers the certainty of deer species identification will dramatically increase. For the rest of the species' examined here, which do not naturally hybridize, a single mitochondrial genetic marker is sufficient for species identification.

In eight of the ten test samples, species origin of the mitochondrial haplotypes amplified was unambiguously typed. In three of these, two mitochondrial haplotypes were amplified from the meat product examined indicating the sample to be of mixed origin. The ability of this system to clearly identify each contributing species will be of great use in forensic research, however, further verification of this technique is required. Blind experiments with known samples need to be conducted to establish the reliability of the system. The effect of DNA quality, that is, average size of DNA fragments, on the likelihood of successful amplification should be tested. Degraded samples with fragments smaller than the average D-loop (~1.2 Kb) may be hard to amplify as the number of complete templates would be significantly reduced. Experimentation should be conducted to establish the sensitivity of this approach for the detection of dual species origin. Experiments controlling the relative amounts of the contributing tissues would establish the minimum contribution needed for detection. Finally, the ability to co-amplify the various D-loop products should be investigated. Single base pair changes in the primer annealing sites or large difference in D-loop product sizes may lead to unequal amplification.

A drawback to this system is its inability to amplify swine and equine D-loop haplotypes. Swine undoubtedly will be a major component in meat products, and in fact was present in the samples F2, F3 and F7 (McClymont, electrophoretic results). However, in wildlife forensic case work it is not the presence or absence of swine that is of interest but the presence of tissue of wildlife origin. The development of swine and equine primer sets would quickly eliminate this drawback.

In two case samples an identical previously unseen haplotype was observed (sample # F4 and F6, Table 3). Due to its overall similarity to the known deer haplotypes this haplotype could tentatively be classified as of deer origin. Estimates of genetic relationship based on RFLP data [*32*] may aid the tentative classification of new haplotypes, however, a definitive match would require a direct comparison with a known haplotype. DNA sequence information would improve the estimate of genetic relationship [*8*]. The discovery of new haplotypes will continue until a larger and

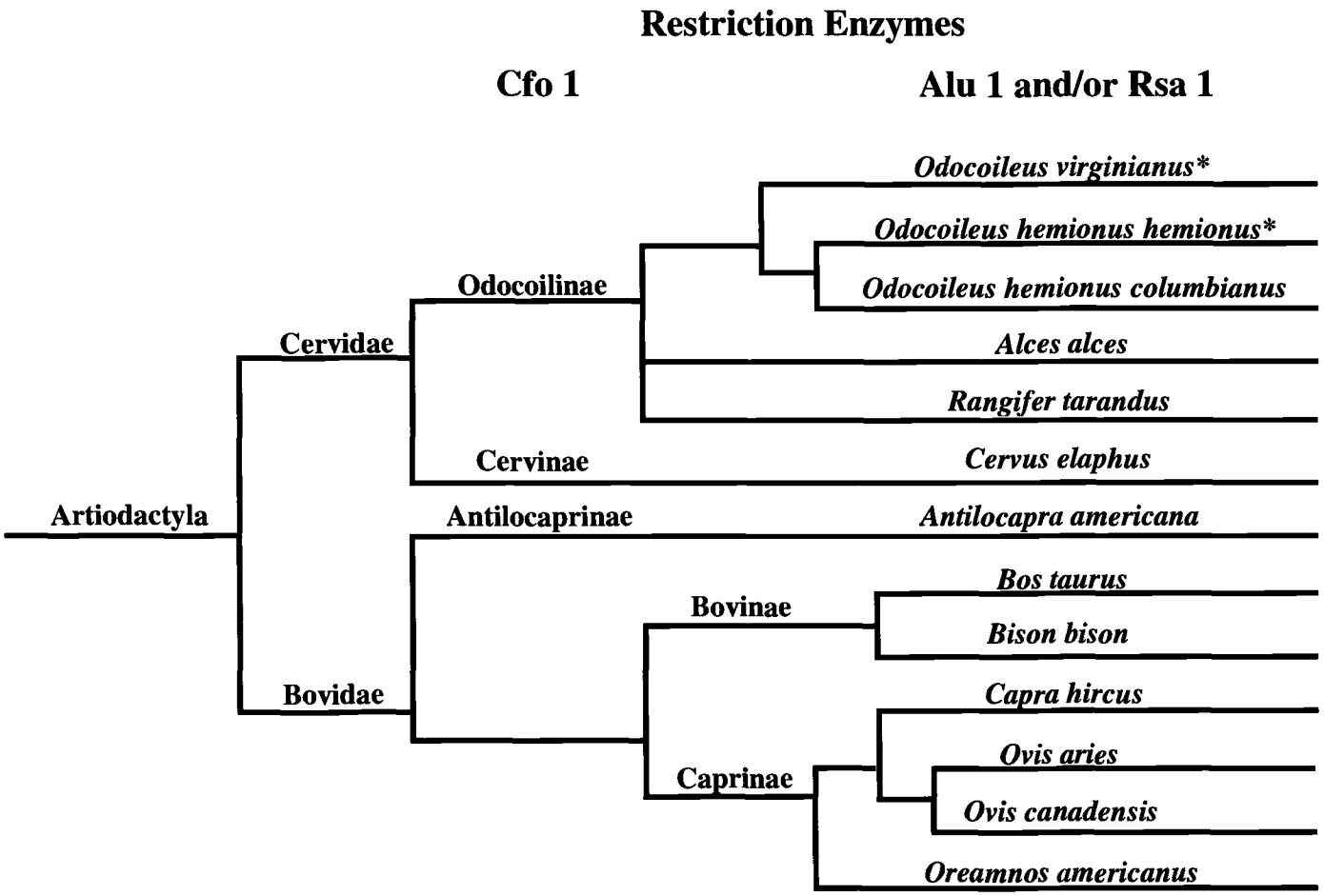


FIG. 3—*Restriction digests of D-loop region of ungulate mitochondrial DNA required to identify species. Cfo 1 distinguishes the subfamilies Odocoilinae and Cervinae in the family Cervidae and the Antilocaprinae from Bovinae and Caprinae in the family Bovidae. The species in these subfamilies can then be distinguished from each other using Alu 1, and/or Rsa 1. Classification as in* [27]. *(*These species share some haplotypes.)*

more geographically complete data base has been developed. For example, in two species, caribou and mountain goat, a number of haplotypes were detected in a relatively small sample (3 in 7 and 4 in 8 respectively). These results suggest that a larger survey would be required to establish the degree of mtDNA variation within these species. Further, for most species only a portion of their geographic distribution was surveyed (such as, Western Canada). Expanding the sample sizes to include new populations from throughout a species range would be required to determine the extent of geographic mtDNA variation. This information would be essential before this technique could be rigorously applied to the identification of most North American ungulates. However, one of the strengths of this technique is that the molecule under examination, the mitochondrial D-loop, is currently being examined in numerous wildlife populations (for example, North American bison and elk results presented here). Thus an excellent data base, independent of the forensic application, is being established.

In addition to expanding the current ungulate data base, the PCR based genetic marker systems developed here to type forensic samples to species could be extended, with or without slight modifications to the primers, to other wildlife groups (for example, carnivores). A major advantage of using D-loop variation is that a number of population surveys of D-loop sequence variation have already been done or are currently being undertaken. Thus a careful and thorough review of scientific journals should be undertaken to keep the data base presented here as complete as possible. Another important objective is to develop a PCR based nuclear DNA marker system for white-tailed, black-tailed and mule deer. The development of nuclear marker systems is essential to establishing species origin between these hybridizing populations.

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