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Forensic Application of Repetitive DNA Markers to the Species Identification of Animal Tissues

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ABSTRACT: Highly repetitive DNA markers have been used for determining the species origin of animal tissues in cases of illegal commercialization and poaching of game animals. This approach has been used in cases involving white-tailed deer (*Odocoileus virginianus*), moose (*Alces alces*) and black bear (*Ursus americanus*). Digesting the DNA with various restriction enzymes, agarose electrophoresis and staining with ethidium bromide revealed unique banding patterns for each species. These patterns have been used to distinguish meat from game animal species from commercial sources of meat and organs. Data are presented from two Ontario court cases that demonstrate the application of the procedure.

KEYWORDS: pathology and biology, species identification, illegal commercialization, white-tailed deer, moose, black bear, repetitive DNA

Identification of the species origin of an unknown sample (blood, tissue, meat, organs) in cases of commercial sales of game animals has typically used protein marker systems [*1–4*]. With a few exceptions [*1*], this technique usually lacks the discriminating power often required to distinguish two closely related species. Also protein electrophoresis is often inadequate for analyzing small amounts of blood or tissues that are not well preserved [*4*]. Techniques involving DNA analysis have recently been developed to provide evidence in wildlife forensic cases [*5–11*]. The stability of DNA allows extraction from partially degraded sources and relatively small amounts of tissue. Also, unlike many proteins, DNA is essentially the same in all cell types within an organism. DNA isolated from forensic samples provides a stable molecule that can be used to address a variety of questions, such as individual identification [*5,10*] and identification of geographical origin [*7*].

Several techniques have been developed using DNA markers to identify the species of an unknown sample. DNA probes for identifying highly repetitive sequences have been cloned from game species to provide species-specific hybridization patterns [*6,9*]. Mitochondrial DNA markers have been used for species identification [*7*]. The polymerase chain reaction (PCR) has been used for species identification by amplifying rDNA

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[*11*] and the amplification and sequencing of the cytochrome b region of mitochondrial DNA [*8*].

We have assessed a rapid and simple method of species identification that uses repetitive satellite DNA sequences. The high copy number of satellite DNA sequences reveal species unique banding patterns following digestion with various restriction enzymes, agarose gel electrophoresis and staining with ethidium bromide. Enzymes have been identified that provide banding patterns that distinguish game animals from each other and from commercial sources of meat and organs.

DNA profiles provide a means of identifying the species origin of tissue samples suspected of being substituted or sold in commercialized meat sales or hunted illegally. We have established species-specific markers for commercial sources of meat such as pork (*Sus domestica*), beef (*Bos domesticus*), lamb (*Ovis aries*) and fallow deer (*Dama dama*), and have determined the species of forensic samples as white-tailed deer (*Odocoileus virginianus*), red deer (*Cervus elaphus*), moose (*Alces alces*), Canadian elk (*Cervus canadiensis*), black bear (*Ursus americanus*) and wild turkey (*Meleagris gallopavo*). Data are presented that were used in two Ontario court cases that demonstrate the application of the procedure.

Materials and Methods

DNA Extraction

Tissue samples (0.4 to 0.8 g) were ground in 3.5 mL 1 × lysis buffer (4 M urea, 0.2 M NaCl, 0.5% n-lauroyl sarcosine, 10 mM CDTA (1,2-cyclohexanediamine), 0.1 M Tris-HCl pH 8.0) (Applied Biosystems Inc.) over liquid nitrogen and then incubated at 37°C for 2 days. Samples were treated with proteinase K (62.5 U, Applied Biosystems Inc.) at 37°C for 12 hours and then extracted twice with phenol:chloroform (70:30) and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated in 0.1 M sodium acetate pH 5.5 by the addition of 1 volume of isopropanol. The DNA precipitate was centrifuged at 7000 g for 20 to 30 min, washed with 70% ethanol. The resultant pellets were dissolved in 250 to 500 μL of TNE_2 (10 mM Tris-HCl pH 8.0, 0.1 mM NaCl, 2 mM disodium ethylene diamine tetraacetate·$2H_2O$ (EDTA). We assessed DNA quality and concentration by agarose gel electrophoresis.

Restriction Enzyme Analysis

DNA (1 to 2 μg) was digested with restriction enzymes (5 U/μg) in conditions recommended by the manufacturer (Bethesda Research Laboratories Ltd.). All digestions were treated with RNase (to a final concentration of 0.1 μg/μL) at 37°C for 1 h. Agarose gel electrophoresis was used to separate the DNA fragments after digestion. DNA (1 μg per lane) was electrophoresed through agarose gels ranging from 0.8 to 1.4%, depending on the fragment sizes to be resolved, at 60 V for 3 to 6 h in Tris acetate (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8). Flanking molecular weight size standards; 0.1 μg per lane of the 1 kilobase (kb) ladder (BRL) or 0.2 μg/lane of bacteriophage lamda DNA digested with *Hind*III, were run on each agarose gel. Agarose gels were stained with ethidium bromide (final concentration 2 μg/mL) for 30 min and destained in 1 mM magnesium sulfate for 30 min. Repetitive DNA bands were then visualized with ultraviolet light.

Results

Identification of Repetitive DNA Markers

Repetitive DNA markers were identified in white-tailed deer using 15 restriction enzymes (Fig. 1). Repetitive DNA bands were observed for all enzymes except *Kpn*I. Low molecular weight (≤1.0 kb) repetitive DNA bands were revealed with digestion by *Hae*III, *Hin*fI, *Msp*I, *Pst*I, *Pvu*II, *Tag*I and *Xho*I. Digestion with *Ava*I, *BamH*I, *Cla*I, *Eco*RI, *Hinc*II, *Hind*III, *Pvu*II, and *Xba*I produced high molecular weight (>1.0 kb) bands. Repetitive DNA bands produced by *Xho*I were used to distinguish white-tailed deer from other cervid species and commercial species.

Distinct repetitive DNA banding patterns were seen in moose with each of the 15 restriction enzymes (Fig. 2). Again, certain restriction enzymes produced lower molecular weight repetitive bands (*Ava*I, *Hae*III, *Hin*fI, *Msp*I, *Taq*I, and *Xho*I) whereas other enzymes generated larger bands (*BamH*I, *Cla*I, *Eco*RI, *Hinc*II, *Hind*III, *Kpn*I, *Pvu*II, and *Xba*I). A prominent band (~0.95 kb) and ~1.0 kb ladder in the *Pst*I digestion was used to distinguish moose from other cervid and commercial meat-providing species. Caribou was also observed as having a ~0.95 kb band and ~1.0 kb ladder (data not shown), however, additional repetitive bands in *Pst*I digested moose DNA distinguished these two species, as did digestion with other restriction enzymes, such as *Xho*I and *Xba*I. Tissue suspected of being from white-tailed deer or moose was analyzed with at least *Pst*I and *Xho*I to provide conclusive proof of the species.

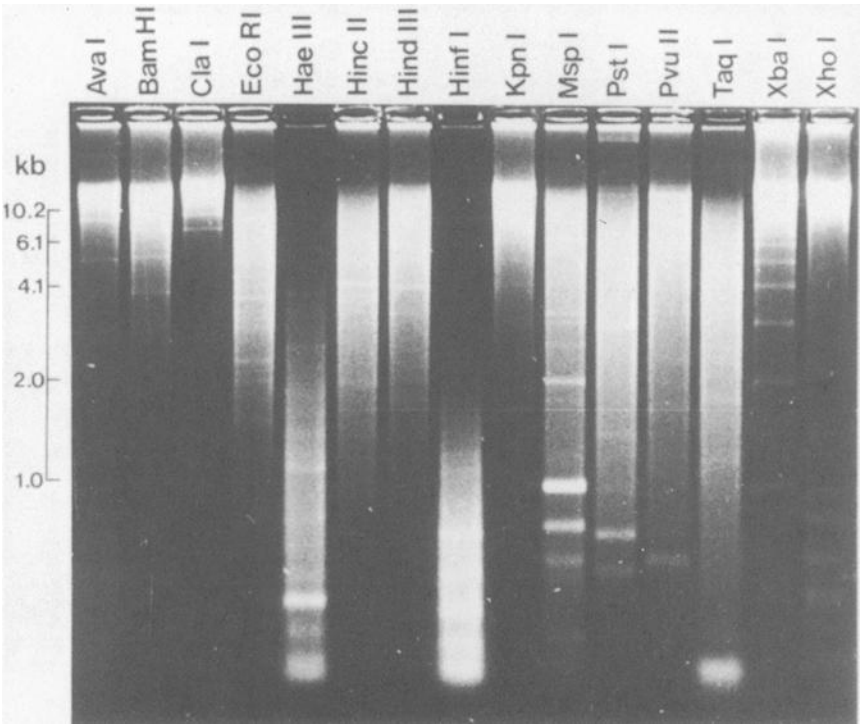

FIG. 1—*Ethidium bromide stained agarose gel (1.2%) of 15 different restriction enzyme digestions of DNA from white-tailed deer (1 μg/lane). The flanking ladder shows the size of bands in kilobases (kb).*

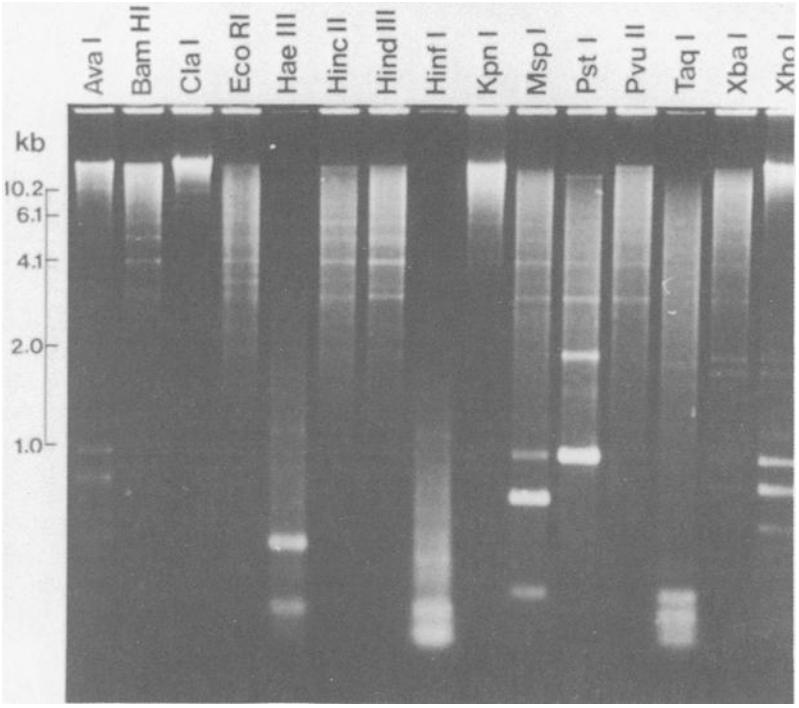

FIG. 2—*Ethidium bromide stained agarose gel (1.2%) of 15 different restriction enzyme digestions of moose (1 μg/lane). Sizes are indicated in kilobases (kb).*

Southern blot analysis of digested cervid DNA hybridized with a mitochondrial DNA (mtDNA) probe revealed a band migrating at ~21 kb (data not shown) which was visible with ethidium bromide staining in some restriction enzyme digests, such as *Pst*I digested moose and white-tailed deer DNA. This band was determined to be uncut mtDNA. No digested mtDNA fragments were observed in the visual analysis of ethidium bromide stained samples.

Southern blot analysis of digested cervid DNA hybridized with a nuclear rDNA probe indicated the banding patterns visible with ethidium bromide staining were not rDNA sequences (data not shown).

Case Applications

One investigation involved illegal sale of moose meat. The two unknown tissue samples were analyzed with controls of beef and moose, white-tailed deer, red deer and elk DNA using the enzymes *Pst*I and *Xho*I (Fig. 3). The 0.95 kb band and ~1 kb ladder in the unknown samples (U1 and U2) matched those in the moose control sample (Aa1). The banding patterns of *Xho*I confirmed these results.

A similar case involved an investigation into the illegal sale of white-tailed deer meat. Three unknown samples (U1, U2 and U3) were analyzed with beef (Bdo), lamb (Oar), moose (Aa1) and white-tailed deer (Ovi) DNA digested with *Pst*I and *Xho*I (Fig. 4). The banding pattern in the unknown samples produced by *Xho*I matched the white-tailed deer control and were confirmed with *Pst*I.

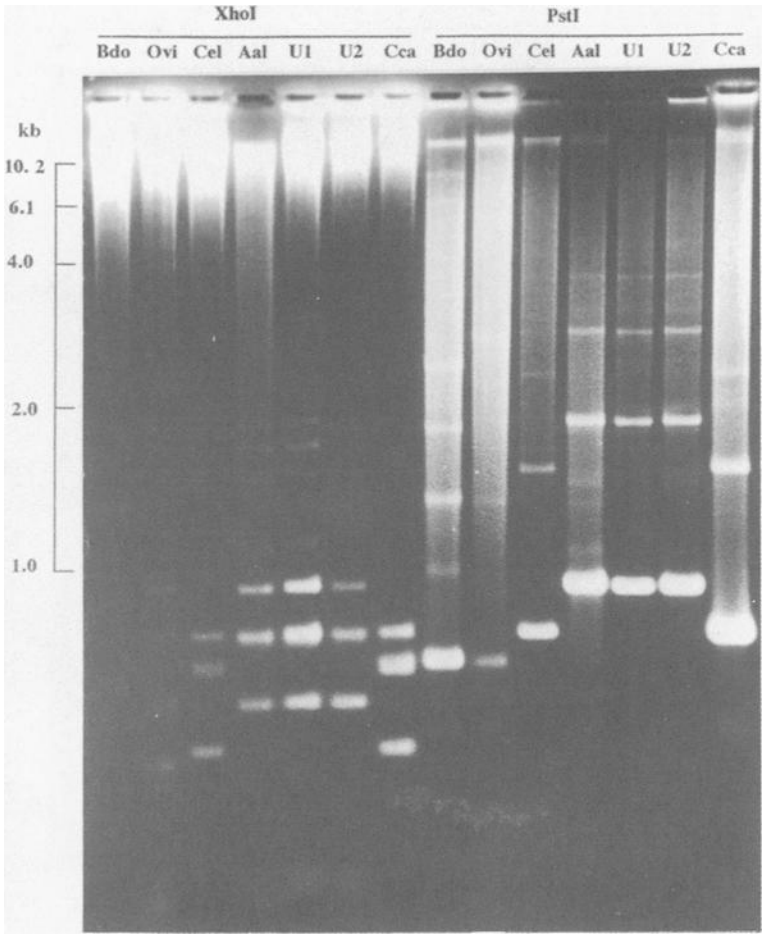

FIG. 3—*Ethidium bromide stained agarose gel (1.0%) of DNA (2 μg/lane) digested with* PstI *and* XhoI *from beef (*Bos domesticus, *Bdo), white-tailed deer (*Odocoileus virginianus, *Ovi), Canadian elk (*Cervus canadensis, *Cca), moose (*Alces alces, *Aal), 2 unknown meat samples (U1 and U2), and red deer (*Cervus elaphus, *Cel). Sizes are indicated in kilobases (kb).*

Discussion

Samples obtained during the investigation of illegal commercial meat sales or poaching investigations often lack species characteristic parts, such as the hide or antlers. We have shown that species-specific repetitive DNA markers are an effective method for identifying the source of a variety of tissues and blood. We have used this technique to identify over 200 tissue samples as moose or white-tailed deer, in addition to identifying gall bladders from black bear, and feathers from wild turkey.

DNA from white-tailed deer (Fig. 1), moose (Fig. 2), other game species and commercial species was examined following digestion with 15 restriction enzymes. Diagnostic enzymes were selected on the presence of prominent bands in a configuration that clearly identified the game species from other species, such as differences in the number and sizes of bands. For example, *Pst*I digestion of moose easily distinguished this species from white-tailed deer and commercial species (Figs. 3 and 4), but the *Pst*I digestion of

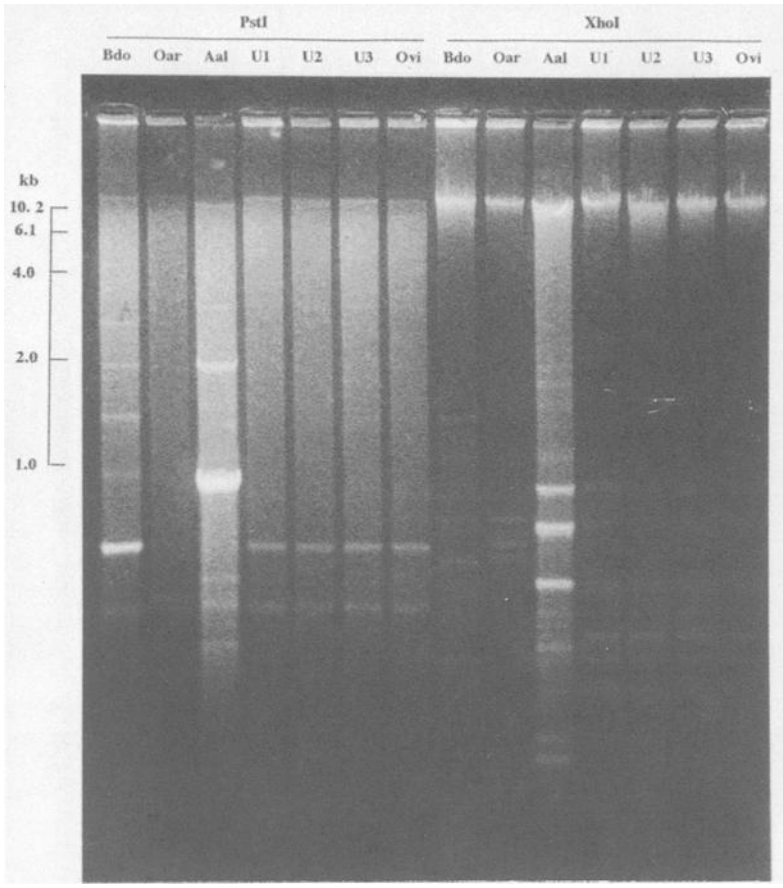

FIG. 4—*Ethidium bromide stained agarose gel (0.8%) of DNA (2 μg/lane) digested with* Pst*I and* Xho*I from beef (Bdo), lamb (*Ovis aries, *Oar), moose (Aal), three unknown samples (U1, U2 and U3) and white-tailed deer (Ovi). Sizes are indicated in kilobases (kb).*

white-tailed deer, beef and lamb revealed similar banding patterns with only an additional two bands to distinguish the three species (Figs. 3 and 4). *Xho*I was selected as the diagnostic enzyme for white-tailed deer because it produced unique banding patterns in those three species. In most cases two restriction enzymes were sufficient for species identification. Since our investigations typically examined illegal sales and poaching of moose or white-tailed deer, the restriction enzymes *Pst*I and *Xho*I were selected to provide a definitive identification of the species origin of meat samples from these two species.

Samples that yield poor quality DNA are not precluded from this type of analysis. Restriction enzymes that produce lower molecular weight repetitive DNA bands can be used to determine the species of an unknown tissue sample. *Hae*III, *Hinf*I, and *Taq*I all produce smaller bands than those produced with other restriction enzymes (Figs. 1 and 2) and are useful when only lower molecular weight DNA samples are extracted. This approach is particularly useful in analyses of gall bladders where the extracted DNA was often in the form of small fragments.

We have analyzed 64 white-tailed deer samples and over 175 moose samples with

*Pst*I and *Xho*I. The majority of white-tailed deer samples were obtained from distinct populations from different geographical regions in Ontario and several samples were obtained from Alberta. The moose samples we have analyzed were obtained from British Columbia (10 samples), Alberta (approximately 25 samples), Ontario (approximately 100 samples) and New Brunswick (10 samples) and have represented two sub-species of moose, *Alces alces andersoni* and *Alces alces americana*. No intraspecific variation was observed in the repetitive DNA banding patterns of the samples we examined.

Species-specific repetitive DNA sequences have been identified in a variety of birds and mammals [*12–15*]. Characterization of species-specific repetitive DNA sequences in cervids [*6,9,16*] has demonstrated that the banding patterns visible with ethidium bromide staining are highly repetitive satellite DNA sequences. Little variation within a species will be observed for repetitive satellite DNA sequences because concerted evolution results in the homogenization of variants within families of repetitive sequences and within a species [*17*]. For this reason repetitive satellite DNA sequences demonstrate a pattern of intraspecific homogeneity and interspecific heterogeneity that is ideal for species identification. Restriction enzyme analysis of repetitive DNA examines species-diagnostic sequence variants, such as differences in the size and sequence of tandemly repeated units between species. To establish species-diagnostic repetitive DNA banding patterns in a game species or protected wildlife species a battery of restriction enzymes, such as 15 restriction enzymes, should be used to compare the species of interest to other species associated in the context of common violations, such as a game animal species compared to various domestic species. Restriction enzymes can be identified which reveal prominent banding configurations, demonstrate interspecific banding patterns and demonstrate low molecular weight banding patterns for use in degraded DNA samples. To confirm the repetitive DNA banding patterns are constant within a species 10 to 20 samples from several geographically distinct populations should be analyzed.

Several techniques using DNA markers for species identification have been developed. Isolated repetitive DNA bands from game species used as probes use radioactive molecular hybridization to identify highly repetitive DNA markers [*6,9*]. This technique is sensitive to very low concentrations of DNA but is time consuming. Mitochondrial DNA (mtDNA) analysis reveals diagnostic mtDNA fragment patterns for the species identification of large mammals [*7*]. Intraspecific variation of mtDNA can be problematic in closely related species, but may be advantageous in the identification of the geographical origin of a sample. PCR amplification and sequencing of the cytochrome b region of the mtDNA is useful for samples with extremely low yields and in cases of game animal meat mixed with commercial meat [*8*]. PCR amplification of variable and conserved regions of the 28S rDNA coding region [*11*] is a potentially useful technique because it will provide results from extremely low yields of DNA, however, it presently has not been applied to game animals and wildlife forensic cases.

The technique we have described is a straightforward method of species identification that is less expensive and faster than the other species identification techniques using DNA analysis. The analysis of extracted DNA using diagnostic restriction enzymes can produce results in less than 8 hours. Analyses requiring probe labeling, such as mtDNA analysis [*7*], hybridization with isolated repetitive DNA (Waye and Haigh 1991, Brackett and Keim 1992) and sequencing [*8*], require 3 to 7 days to produce results from extracted DNA. The initial costs of establishing a species identification facility are considerably less expensive for the technique we describe because equipment such as a thermocycler and sequencing apparatus are not required. Also the cost per sample of analysis for sequencing and radioactive labeling is higher than the cost per sample of analyzing visible repetitive bands.

One disadvantage of visual assessment of repetitive DNA bands in ethidium bromide stained samples is that analysis is not possible for low yields of DNA [*6,9*]. In our

experience with wildlife forensic cases the amount of DNA extracted from minute samples, such as blood stained clothes and knives, is in the range of 5 to 10 μg, which is sufficient for analysis. Smaller amounts can be further analyzed by Southern blotting and applying one of the techniques using DNA probes, such as mtDNA probes [*7*] or repetitive DNA probes [*6,9*] to increase sensitivity. Another disadvantage with the analysis of visually assessing repetitive DNA bands occurs in mixed meat samples of game and commercial species [*6*]. These samples would benefit from Southern blot analysis using DNA probes.

Wildlife forensic science is beginning to receive more technological attention. Although only about 1% of wildlife crimes result in charges being laid, 98% of charges result in a conviction. All the cases involving repetitive DNA markers for species identification we have presented in Ontario provincial court have been accepted as evidence.

Acknowledgments

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