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Application of DNA Fingerprinting to Enforcement of Hunting Regulations in Ontario

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ABSTRACT: DNA fingerprinting has been used in investigations of 40 cases of infractions of hunting regulations involving white-tailed deer (*Odocoileus virginianus*) and moose (*Alces alces*) in Ontario. In most of these cases, individual-specific DNA fingerprints obtained with the Jeffrey's 33.15 multilocus probe were used to link the animal remains found at the illegal kill site to blood and tissue samples of the dead animal associated with a suspect. DNA fingerprints from 27 white-tailed deer and 19 moose were obtained in order to establish the level of band-sharing in DNA fingerprints among unrelated individuals in each species. We also determined the levels of band-sharing among animals from the same region and calculated the probability of two individuals sharing the same DNA fingerprint. Details are presented from cases in which the evidence was presented and accepted by Ontario courts.

KEYWORDS: forensic science, pathology and biology, DNA fingerprinting, white-tailed deer, moose, poaching

The advent of DNA fingerprinting [*1,2*] has allowed the identification of individuals from DNA extracted from tissues, blood, semen, and hair roots, and has proved a vital factor in many human forensic investigations [*3,4,5*]. It has been proposed that DNA fingerprinting could be a powerful tool in the enforcement of Game and Fish regulations [*6*]. DNA fingerprinting can link a suspect to a kill site of an illegally hunted animal by matching blood or tissue from the dead animal at the kill site to blood or tissue associated with the suspect.

The initial requirement before DNA fingerprinting can be applied to enforcement is a data base of DNA fingerprint banding patterns from unrelated individuals of the species in question. These types of data bases have been established for use in human forensic investigations for both single locus and multilocus DNA fingerprint probes [*7,8*]. A primary purpose of establishing DNA fingerprint data bases is to obtain a conservative estimate of the probability of two animals sharing the same DNA fingerprint. Comparisons of DNA fingerprints among unrelated individuals provides an estimate of band sharing. Average levels of band-sharing in noninbred populations have been found in the range of 0.2 to 0.3. Factors that increase the average level of band-sharing, such as population structure [*9*] and linkage among DNA fragments [*10*], must be examined.

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Without careful consideration of these factors, the levels of band-sharing may be inaccurately estimated and the probability estimates of a random match of the DNA fingerprints would be incorrect [*11*,*12*].

We have applied DNA fingerprinting to 40 cases involving white-tailed deer and moose poaching in Ontario. DNA fingerprint data bases have been assembled for both species using the human multilocus 33.15 probe [*1*] in order to establish the probability of obtaining identical DNA fingerprints from two unrelated animals. Here, we present the application of this technique to four cases.

Materials and Methods

DNA Extraction

Tissue samples (0.5 to 0.8 g) were ground in liquid nitrogen with 3.5 mL lysis buffer (4 M urea, 0.2 M sodium chloride, 0.5% n-lauroyl sarcosine, 10 mM CDTA, 100 mM Tris-HCl, pH 8.0; Applied Biosystems Inc.). Small pieces of clothing stained with blood (approximately 5 cm^2) were added to 3.5 mL lysis buffer. All samples were incubated at 37°C for up to one week. Proteinase K (62.5 U; Applied Biosystems Inc.) was added and each sample was incubated at 37°C for an additional 4 to 7 days. DNA was purified by two phenol:chloroform (70:30) extractions and one chloroform:isoamyl (24:1) extraction, then precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.5) and 1.0 volume of isopropranol. Precipitates were centifuged at 7000 × g for 30 min, washed with 70% ethanol and recentrifuged. The DNA was dissolved in 250 to 500 μL of 1 × TNE_2 (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA (disodium ethylene diamine tetraacetate·$2H_2O$) pH 8.0). Agarose gel electrophoresis was used to assess the quality and quantities of all samples.

Southern Blotting and Hybridization

Approximately 10 μg of DNA was digested with the restriction enzymes *Alu*I or *Hae*III (10 U/μg DNA) in conditions recommended by the manufacturer (Bethesda Research Laboratories Ltd.), followed by treatment with RNase (to a final concentration of 0.1 μg/μL) at 37°C for 1 hour. Each digestion was extracted and precipitated as previously described and dissolved in 40 μL of distilled water. A 1 μL aliquot of each reaction was run on an agarose test gel (0.8%) to determine the concentration of the digested DNA. Prior to loading, one-fifth volume of a gel loading buffer (0.5% orange g, 15% ficoll, type 400, 50 mM EDTA, pH 8.0) was added and all digestions were heated at 65°C for 15 min. Five micrograms of each digestion were then electrophoresed through a 30 cm long 0.8% agarose gel in Tris borate buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 80 V for 24 to 26 hours. Five ng of a mixture (1:1) of lambda (λ) DNA cut with *Bst*EII and a λ DNA double digest with *Hind*III and *Eco*RI was run simultaneously in each genomic DNA lane to account for any differences in sample migration. A 5 μg *Alu*I digested human sample was run on each agarose gel as a control for the quality of Southern blotting and hybridization. DNA was transferred to Gene Screen Plus nylon membrane (Dupont/NEN Inc.) according to the manufacturer's protocol.

Membranes were soaked in 5 × SSC (10 × SSC: 1.5 M NaCl, 0.15 M sodium citrate) and prehybridized in 25 ml of hybridization buffer (0.26 M Na_2HPO_4, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA pH 8.0, 1% bovine serum albumin) [*13*] at 65°C for 2 to 6 h. We used two probes in our analyses, the Jeffrey's 33.15 minisatellite probe [*1*] and the *per* locus probe [*14*], and bacteriophage lambda DNA. Each probe was radioactively labelled (25 ng each) by the random primer extension method [*15*] with 50 μCi α^{32}P[dCTP] (Dupont/NEN Inc.). Unincorporated label was removed by passing the solution through

a G-50 Sephadex column. Specific activities were typically $> 1.0 \times 10^9$ cpm/μg. Hybridization was carried out at 65°C for 20 to 22 hours. Blots were washed in 2 × SSC, 0.1% SDS; once at room temperature for 15 min, and twice at 65°C, for 15 min then 30 min. After rinsing in 1 × SSC, blots were exposed to Cronex 4 or Kodak XAR X-ray film using one intensifying screen at −70°C for seven days. Southern blots were stripped between hybridizations in 0.4 N sodium hydroxide at 42°C for 30 min, then neutralized in 0.1 × SSC, 0.5% SDS, 0.2 M Tris-HCl (pH 7.5) for 30 min at 42°C.

DNA Fingerprint Analysis

The DNA fingerprints were analyzed using the methods outlined in Galbraith et al. [*16*]. All visible bands from each DNA fingerprint were marked on an acetate sheet. A second acetate was made of each internal size standard. The size of each band was measured using a Grafbar computer-driven sonic digitizer with a subroutine from the Cyborg program (International Biotechnologies Inc.). Each lane was digitized three times and the mean from these three measurements was used. In all comparisons bands less than 2.8 SD apart in size were considered the same, regardless of intensity. Since the error in the digitizing measurements is greater for larger bands, we used different coefficients of variance (CV) to determine the standard deviation (SD) between two bands; depending on the size of the band. Mean CVs for band size ranges of > 20 kb, 10 to 20 kb, 5 to 10 kb, and 2.5 to 5 kb (white-tailed deer) or 2.1 to 5 kb (moose) were used, whereas in Galbraith et al. [*16*] the number bands shared was determined with a constant CV. Band-sharing coefficients (D) between pairs of individuals were then calculated using the formula $2N_{AB}/(N_A + N_B)$ where N_{AB} is the number of bands shared for individuals A and B and N_A and N_B are the total number of bands in individual A and individual B, respectively [*17*]. Band-sharing coefficients for pairs of animals from across the province provided mean overall values for moose and white-tailed deer. D values for groups of animals from the same location were also determined where possible. The band-sharing coefficient used, in the calculation of the probability of two animals sharing the same DNA fingerprint, was 0.50 for white-tailed deer and 0.60 for moose; these values allowed for population substructuring and the possibility of two animals being second degree relatives.

Results

White-tail Deer

The first case in which DNA evidence was presented in Ontario Provincial Court involved the analysis of DNA isolated from a buck's head seized from the suspect and that obtained from blood found in a plastic bag found near the probable kill site. Digestion of both samples with *Hae*III and probing with *per* gave a scorable banding pattern (lanes 1 and 2, Fig. 1). Each of the 22 bands in the DNA fingerprint of the buck's head (lane 1) matched a corresponding band in the DNA fingerprint (lane 2) of the blood from the plastic bag (Fig. 1). The band intensities of the fingerprint of the blood were weaker because of a low yield of DNA. In order to calculate the probability of two animals sharing the same DNA fingerprint, a band-sharing coefficient for unrelated white-tailed deer was determined. DNA from fourteen white-tailed deer from Manitoulin Island was digested with *Hae*III and probed with Jeffreys' 33.15 and *per* (Fig. 2A). In lane lambda markers were used to standardize band scoring (Fig. 2B). DNA fingerprints produced with the *per* probe generally revealed a pattern of weaker hybridizing high molecular weight bands and strongly hybridizing low molecular weight bands (Fig. 2A). Band-sharing coefficient for pairs of individuals ranged from 0.19 to 0.84 (mean = 0.47) for

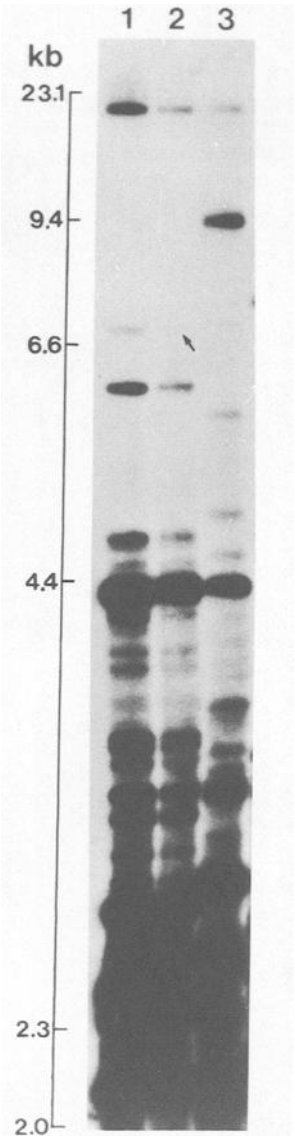

FIG. 1—*Autoradiograph of* Hae*III digested DNA probed with* per *from a white-tailed deer head (1) seized from the poachers, blood from a plastic bag (2) found on an island where hunting was prohibited, and an unrelated white-tailed deer (3). The arrow in lane 2 indicates a faint band that corresponds to the band seen in lane 1.*

the *per* probe. To estimate the likelihood of two animals sharing the same DNA fingerprint a higher band-sharing coefficient for the *Hae*III/*per* combination of 0.60 was used to allow for potential relatedness in a local population. The probability of another deer sharing the 22 bands identified in the buck's DNA fingerprint was therefore estimated at $(0.60)^{22}$ or 1.31×10^{-5}. Therefore the probability that the blood in the plastic bag came from the buck's head found in the possession of the suspect was estimated as being greater than 100 000:1.

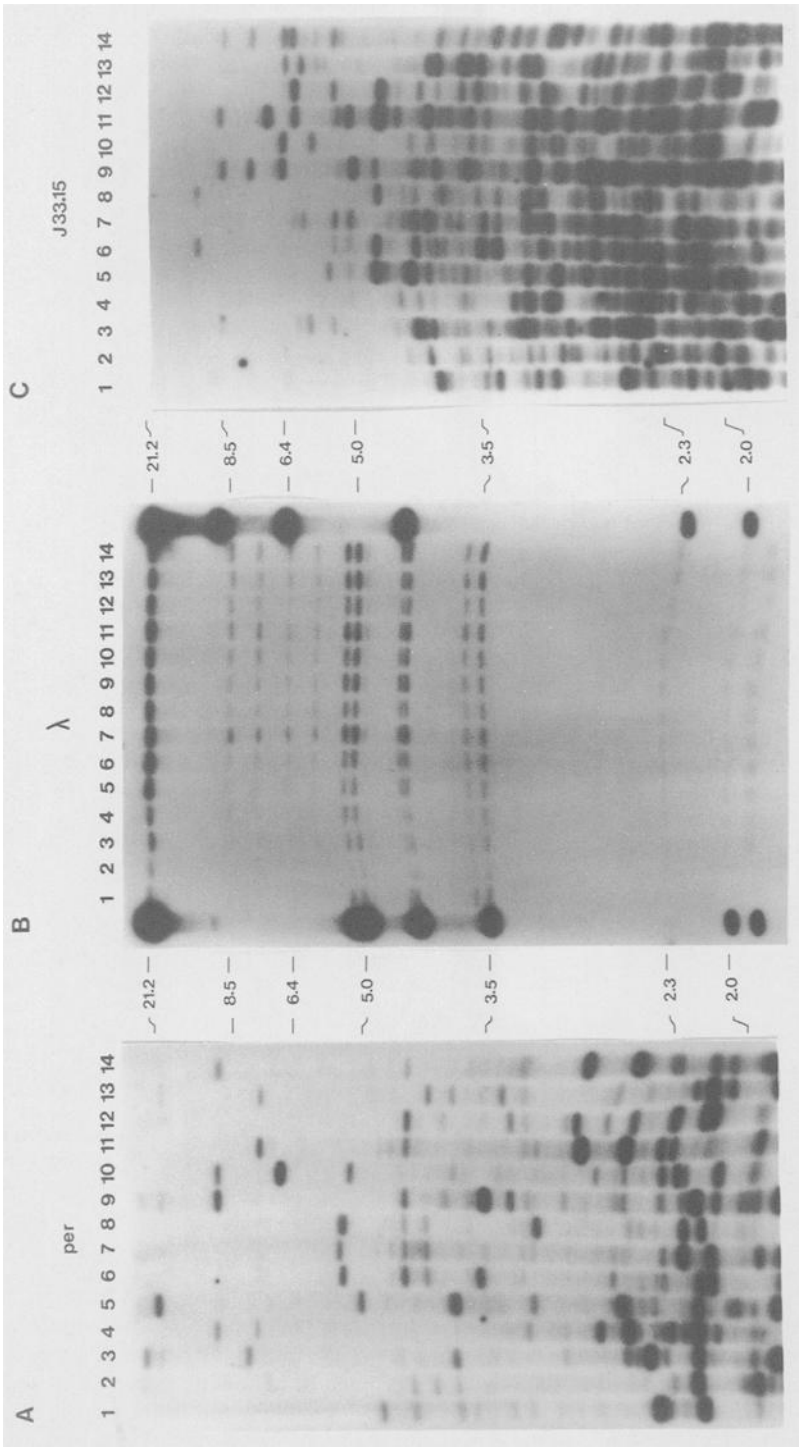

FIG. 2—*Autoradiographs of* Hae*III digested white-tailed deer DNA (5 μg per lane) probed with the* per *locus (A), internal size standards probed with lambda (λ) DNA (B), and* Hae*III digested white-tailed deer DNA probed with Jeffrey's 33.15 (C) from 14 different individuals from Manitoulin Island, Ontario. Samples were electrophoresed through a 0.8% agarose gel, blotted, and hybridized with the indicated probe. Specific activities were 1–2.0 × 10⁹ cpm/μg.*

The Jeffreys' 33.15 probe identified fingerprints comprised of more bands and showing a lower mean band-sharing coefficient than the *per* probe in animals from Manitoulin Island (Fig. 2C). We therefore chose to examine the mean band-sharing coefficient for the 33.15/*Hae*III combination from animals across the province (Table 1). For the 27 animals analyzed an average of 6 of 22 bands were shared giving a mean D value of 0.29 ± 0.09 (SD). In order to allow for the population structuring suggested by the higher band-sharing coefficient found with the Manitoulin Island population and a higher probability that any two animals in a local population were related, we decided to use a conservative band-sharing coefficient of 0.50 to estimate the probability of two deer sharing the same fingerprint.

In another case, DNA fingerprints derived from bloodstains from a suspect's snowmobile suit were compared to DNA fingerprints of tissue samples from the remains of two bucks and a fawn at the kill site (Fig. 3). The DNA fingerprint from one bloodstain preparation (lane 7) matched the DNA fingerprint of tissue from one kill site (lane 3). The intensity of bands in lane 3 in Fig. 3 is lower than those in lane 7 but on longer exposure 21 resolvable bands were identified for each of these samples. The probability that the DNA fingerprint from the bloodstain in lane 7 was from a different animal was estimated as $(0.50)^{21}$ or 4.76×10^{-7}. The DNA fingerprints from the other bloodstains (lanes 4 to 6) had a greater number of bands than those from tissues at the kill sites (lanes 1 to 3). The patterns and band intensities, revealed that these bloodstains were probably composed of blood from more than one animal. Each band in the DNA fingerprints of the bloodstain preparations in lanes 4 and 6 matched a corresponding band in either the DNA fingerprint of the first buck (lane 2) of the second buck (lane 3). All bands in the DNA fingerprint of the remaining bloodstain (lane 5) matched bands from the fawn (lane 1) or the first buck (lane 2).

Moose

Moose DNA was digested with *Alu*I and *Hae*III and hybridized with both the *per* and Jeffreys's 33.15 probes (Fig. 4). The *Alu*I Jeffreys's 33.15 derived fingerprints gave the

TABLE 1—*Band-sharing coefficients among white-tailed deer and moose from Ontario using the Jeffreys's 33.15 multilocus probe.*

Species	Band Size Range (kb)	No. of bands/individual (± SD)	Band-sharing frequency (D^a ± SD)
		White-tailed deer	
(N = 27)	>20	0.2 ± 0.42	0.47 ± 0.52
	10–20	1.1 ± 1.05	0.16 ± 0.27
	5–10	4.2 ± 2.37	0.17 ± 0.18
	2.5–5	16.1 ± 2.23	0.33 ± 0.11
	Mean	21.7 ± 3.55	0.29 ± 0.09
		Moose	
(N = 19)	>20	0.2 ± 0.36	0.33 ± 0.58
	10–20	1.2 ± 0.93	0.14 ± 0.27
	5–10	2.0 ± 0.73	0.28 ± 0.30
	2.1[b]–5	20.4 ± 2.87	0.45 ± 0.15
	Mean	23.7 ± 3.31	0.42 ± 0.14

[a]The band-sharing frequency (D) was calculated from $2N_{AB}/N_A + N_B$, where N_{AB} is the number of bands shared by individuals A and B and N_A and N_B are the total number of bands in individual A and B, respectively (Wetton et al).

[b]A larger band size range was examined in moose to obtain a greater number of informative DNA fingerprint bands.

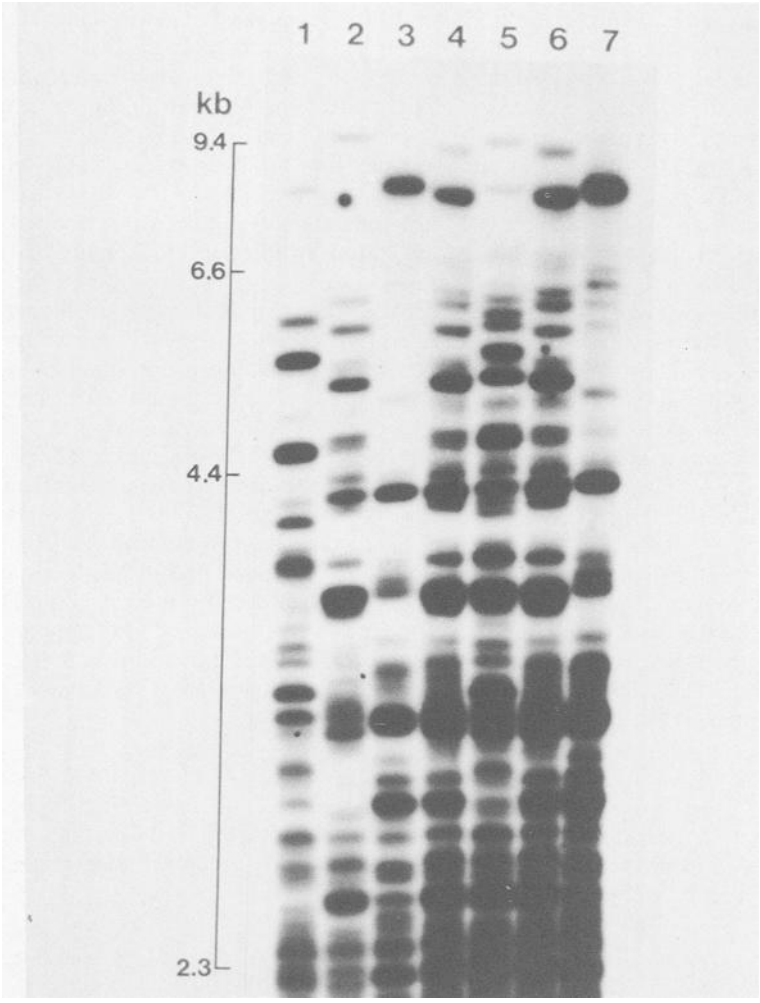

FIG. 3—*Autoradiograph of* Hae*III digested DNA probed with Jeffreys's 33.15 from 3 white-tailed deer found at the kill site; a fawn (1) and two bucks (2 and 3), and four different preparations from the suspect's blood-stained snowmobile suit (4–7).*

clearest patterns and this combination was used to establish the mean level of band sharing (Table 1). The 19 unrelated moose drawn from across the province had an average of 24 scorable bands with a band-sharing coefficient of 0.42 (Table 1). This mean band-sharing coefficient is significantly higher than that found for white-tailed deer with 33.15/ *Hae*III (0.29). As with the white-tailed deer we found that pairs of animals from the same geographic region had slightly higher band-sharing coefficients. In order to allow for population structuring and the possibility that two animals in one location were related we used a band-sharing coefficient of 0.60 to estimate the probabilities of two different animals sharing the same DNA fingerprint.

In one case, DNA fingerprints (Fig. 4) from a tissue sample of remains of a bull moose (lanes 2 and 8) at the kill site were compared to those from a meat sample (lanes 2 and 9) from the suspect's freezer. In both the *Alu*I and *Hae*III digested DNA probed with Jeffrey's 33.15, all of the bands from the tissue at the kill site matched those derived

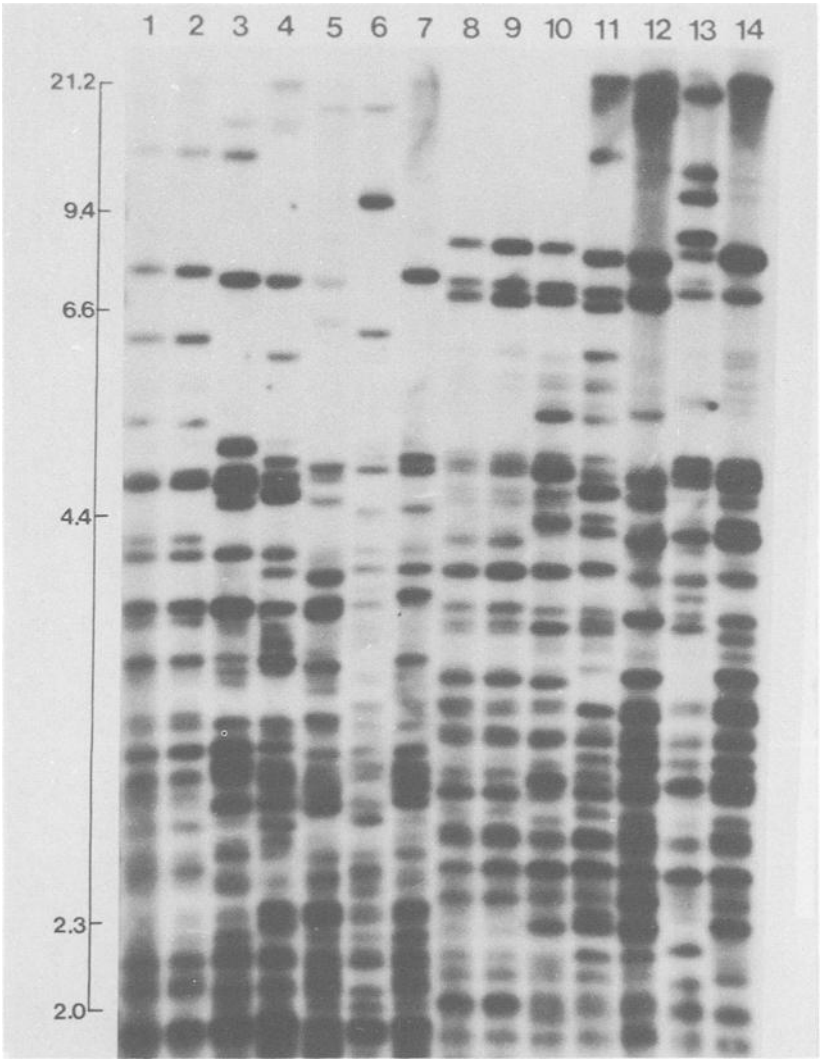

FIG. 4—*Autoradiograph of* Alu*I (1–7) and* Hae*III (8–14) digested moose DNA probed with Jeffreys's 33.15. Lanes 3–7 and 10–14 represent five different animals from across Ontario. Lanes 1 and 8 were from tissue obtained from the remains of a bull moose at a kill site and lanes 2 and 9 were from a meat sample from the suspect's freezer.*

from the meat in the freezer. The number of bands scored in the *Alu*I digest was 22 and therefore the probability that these fingerprints were derived from two different animals was estimated as $(0.6)^{22}$ or 1.31×10^{-5}.

The second case involved a group of hunters suspected of poaching five moose. DNA fingerprints were obtained from three tissue samples found in their vehicle and trailer, two liver samples from a freezer and tissue from the remains of an adult bull and yearling bull at the kill sites (Fig. 5). The 23 bands found in the DNA fingerprint from the remains of the yearling bull moose matched those of the two liver samples obtained from the suspect's freezer (lanes 2, 3, 4). This comparison emphasizes the importance of the standard markers in each lane. The bands in the fingerprint from the yearling bull moose

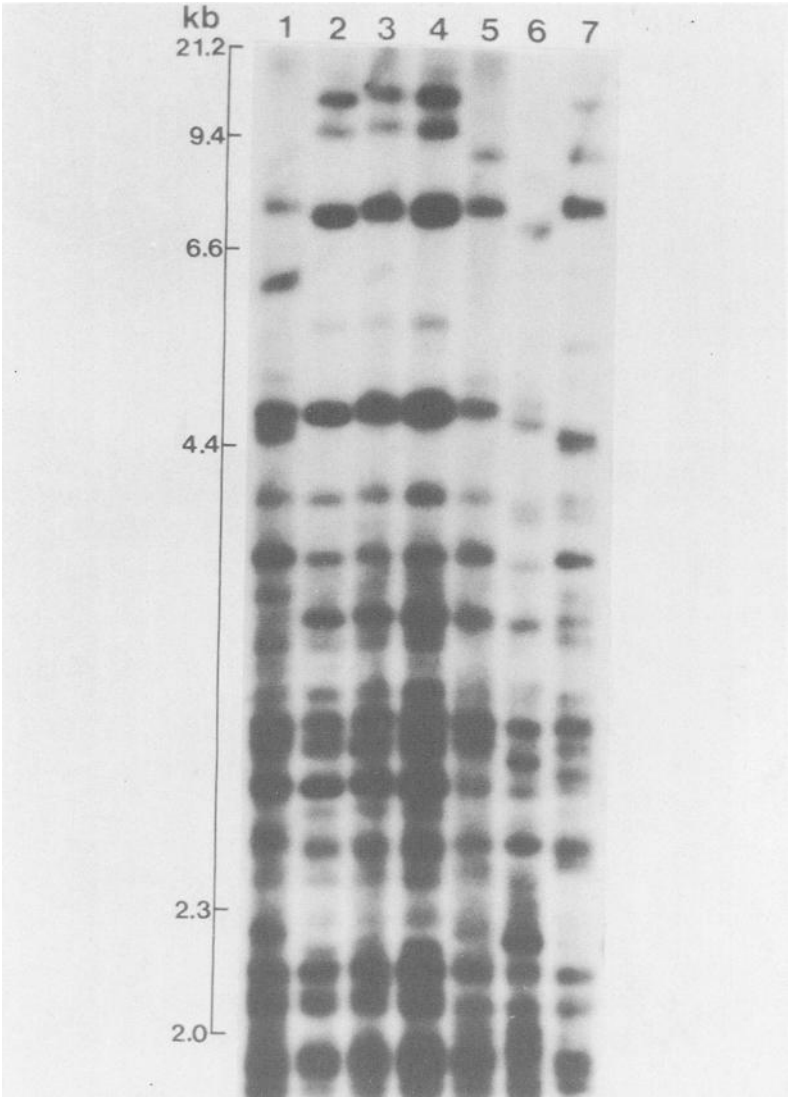

FIG. 5—*Autoradiograph of* Alu*I digested moose DNA probed with Jeffrey's 33.15 from an adult bull (1), a yearling bull carcass (2), two liver samples (3 and 4), a tissue sample found in a hunter's vehicle (5) and two tissue samples from the hunters' trailer (6 and 7).*

(lane 2) migrated faster than those from the liver (lanes 3 and 4). When these were assessed using the internal lambda markers the sizes of all 23 bands matched. The probability that the liver came from an animal other than the yearling bull moose found at the kill site was estimated as $(0.60)^{23}$ or 7.89×10^{-6}. The DNA fingerprints obtained from the tissues found in the vehicle and trailer (lanes 5 to 7) represented three additional animals. Thus the DNA fingerprints confirmed the suspicions of the conservation officers that five animals had been killed.

Discussion

DNA fingerprints revealed by the hybridization of minisatellite DNA probes to white-tailed deer and moose DNA have provided important evidence in a number of cases in Ontario. They have usually been used to establish the link between evidence obtained from a suspect to tissue found at kill sites. Many of these cases of hunting violations were initially discovered by locating a gutpile or blood sample at the kill site. The DNA fingerprint linked these tissues to: blood found on clothes, knives, or on the suspect's vehicle; meat found frozen in the suspect's residence; the head and/or antlers possessed by the suspect. In several cases a good quality DNA sample was obtained from slightly degraded tissues. However, these samples often provide a low yield of DNA resulting in weaker bands on the autoradiographs (for example, lanes 3 and 7 in Fig. 3).

The probability of two different animals sharing an identical DNA fingerprint is calculated by D^x, where D is the band-sharing coefficient and x is the number of bands in the fingerprint. The D values we used in these estimates were modified from the mean band-sharing coefficients from animals across the province to take into account population structuring and potential relatedness of animals from the same region. A debate over the need for such a consideration of population structuring has arisen for human forensic applications [*8,12,19,20*]. We have adopted a conservative approach by using a D value considerably higher than the mean found for animals across the province. The definition of conservative with respect to courtroom application is when, on average, the estimate of the probability is greater than the actual probability so that the estimate favors the suspect [*21*]. We are confident that the D value we have used provides a reasonable estimate that maintains the integrity of the statistical analysis to provide definitive evidence of a match with no bias against the suspect.

The assessment of DNA fingerprints requires screening various enzyme/probe combinations to derive the banding patterns that provide the highest mean number of scorable bands and the lowest mean band-sharing coefficient. This provides the DNA fingerprint that will demonstrate the most variability and therefore the better statistical evaluation of a match.

We recommend the *Hae*III/Jeffreys' 33.15 combination for white-tailed deer and the *Alu*I/Jeffreys' 33.15 combination for moose for the respective DNA fingerprints. Using *Hae*III/33.15 we estimated a mean band-sharing coefficient of 0.29 for white-tailed deer from animals across the province and a mean value of 0.37 for animals in one region. We have thus used the conservative value of 0.50 to estimate the probability of two different white-tailed deer having the same DNA fingerprint. The mean band-sharing coefficient for moose drawn from across the province was 0.42 with *Alu*I/33.15. With local populations having a slightly higher value, we used a band-sharing coefficient of 0.60 to calculate the probability of two moose sharing the same DNA fingerprint.

A higher level of band-sharing was found for moose than for white-tailed deer (Table 1). This may reflect the history of the two species and supports the idea of a population decline of moose as a result of the recent ice-ages. Mitochondrial DNA analyses [*22*] and protein marker studies [*23*] have also shown lower levels of genetic variation in moose in comparison to other cervids.

The data presented in this paper are from moose and deer enforcement cases as they are the most frequent wildlife violations requiring DNA fingerprinting in Ontario. DNA profiling has broader applications to wildlife management. Illegal hunting of birds, fish, and protected animals can also use DNA technology providing the samples are present to make a match between the appropriate tissues. The control of the illegal exporting of endangered species caught in the wild can also benefit from DNA fingerprinting because parentage analysis can provide exclusion of an individual animal from the captive stock used for the breeding of legally exported animals.

To be useful for conservation officers DNA profiling must be relatively inexpensive and the results should be available in less than four weeks. We recommend that sample preparation in the field involve freezing the blood or tissue as soon as possible, or preferably to place any material in vials containing a preservative buffer (4 M urea, 0.2 M sodium chloride, 0.5% n-lauroyl sarcosine, 10 mM CDTA, 100 mM Tris-HCl pH 8.0; Applied Biosystems Inc.) or pickling solution (DMSO: 20% dimethyl sulfoxyle, 0.25 EDTA, pH 8.0). The DNA in tissues preserved in these solutions is stable for months at ambient temperature and can be easily transported to the forensic laboratory.

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