APPLICATIONS OF DNA TECHNOLOGY TO WILDLIFE FORENSIC SCIENCE

By

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ABSTRACT

Molecular genetic protocols have been developed to provide evidence in infractions of wildlife statutes in Canada. We have utilized DNA marker systems to address specific questions in wildlife investigations based on their different levels of genetic variability. Multilocus DNA fingerprinting has been applied to poaching infractions to determine if tissue samples associated with a suspected poacher originated from the remains of an animal at a known illegal kill site. The hypervariability of the variable number of tandem repeat (VNTR) loci detected by multilocus DNA fingerprinting allows the individual identification of samples. Highly repetitive satellite DNA markers have been applied to determining the species of origin of unknown tissue samples based on their species-specificity. Satellite DNA profiling have provided evidence in illegal commercialization investigations involving species such as moose (*Alces alces*) and white-tailed deer (*Odocoileus virginianus*), including the illegal addition of game meat in processed meat products. A sex-specific DNA locus, the sex-determining region on the Y-chromosome (Sry), has been utilized to determine the sex of cervid samples that have had gender-specific physical characteristics, antlers and genitalia removed in violation of the validation tag system. Finally, a polymerase chain reaction (PCR) based protocol has been established for the species identification of samples that produce minute amounts of DNA or degraded DNA. Cytochrome b sequences

demonstrate low intra-specific levels of sequence divergence and higher inter-specific levels of sequence divergence. Cytochrome b sequence analysis has been applied to fish, game and domestic species commonly involved in wildlife investigations and to the identification of a number of species, mostly seal species, involved in the trade of animal parts.

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I would like to first thank Dr. Bradley N. White for teaching a Maritimer to write good English, at least partially, and for teaching me how to think both critically and scientifically.

I also thank my committee, Dr. Jim Quinn and Dr. John Waye, for giving me the time to read and evaluate my thesis and knowledge.

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Finally, my gratitude to my parents: Mom and John who kept the thesis on the forefront of my thoughts since the day I started. I thank them for behaving as parents despite my age and distance. My brother and sister also receive my thanks; home felt a lot less far away with their support and visits .

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PREFACE

Chapters one to six represent manuscripts that have been published or submitted for publication. My role in the manuscripts that have additional authors to Dr. White and myself is as follows:

CHAPTER ONE

The initial DNA fingerprinting of samples was performed by E. Guglich. I contributed significantly to the writing of the manuscript and providing input into the analysis of the data.

CHAPTER TWO

The initial data was collected by E. Guglich. I followed-up on the data by providing additional testing to determine the markers were highly repetitive DNA and I provided one of the case examples for the manuscript. I also contributed significantly to the writing of the manuscript.

CHAPTER FIVE

Two fourth year project students provided several cytochrome b sequences for the study.

CHAPTER SIX

Miss Malik performed the technical work under my direction in the laboratory. I analysed the data and wrote the manuscript with R. Smith, Dr. Lavigne and Dr. White.

GENERAL INTRODUCTION

The majority of hunters and anglers comply with the Fish and Game regulations of the provincial and federal governement agencies responsible for the management of Canadian natural resources. However, there are those individuals who violate these regulations with a total disregard for the conservation of the animal life for which they exploit. The act of poaching is the illegal taking of wildlife and is defined as "any animal that has been taken by action or omission that contravenes the acts and regulations made to conserve wildlife" (Gregorich 1992). Two types of poachers exist: the first type participate in illegal hunting practices for personal consumption; and the second type of poacher partakes in the illegal commercialization of fish and game meat or the trade of animal parts for profit.

Conservation officers and park wardens must utilize every tool available to apprehend and convict poachers and for this reason wildife forensic technology has been modeled after the developments of human forensic science. Prior to the mid-1980's, biological evidence utilized serological or protein based techniques to provide evidence in both human and wildlife investigations. The focus in human investigations was the individual identification of tissues associated with a crime using ABO or human leukocyte

antigen (HLA) typing (Howlet 1989), while wildlife investigations utilized allozymes to determine the species of an unknown tissue sample (Bunch 1976, McClymont 1982, Wolfe 1983, Mardini 1984, Pex and Wolfe 1985). Protein-based techniques utilized for individual identification in human forensic analysis and limited attempts in wildlife forensic science (Pex et al. 1985, Thommasen et al. 1989) offered relatively weak statistical associations or probable exclusions. The development of DNA "fingerprinting" by A.J. Jeffreys and his colleagues in 1985 (Jeffreys 1985) revolutionized both human and wildlife forensic science.

DNA "fingerprinting" utilizes regions of the genome called minisatellite DNA (Jeffreys et al. 1985) which are tandemly repeated nucleotide sequences of approximately 50 b.p. Minisatellite loci are hypervariable as the result of high mutation rates that generate new alleles that differ in the number of repeat units in a tandem array (Jeffreys et al. 1985, Wolff et al. 1989, Jeffreys 1994) and these regions of DNA have been appropriately termed variable number of tandem repeat (VNTR) loci (Nakamura et al. 1986). VNTR arrays contain a "core" sequence of 10-15 b.p. that is present in the repeat units of memebers of a satellite DNA family (Jeffreys et al. 1985, Nakamura et al. 1987). Hybridization with a probe containing this "core" sequence allows the simultaneous detection of a large number of VNTR loci and estimates of 60 loci have been detected using the two minisatellite probes Jeffreys 33.16 and 33.6 (Jeffreys et al. 1986). This multilocus detection results in an individual-specific DNA "fingerprint".

Advances in molecular genetic techniques were also being made in establishing DNA markers to examine genetic variation and address biological questions in natural populations of animal species. Single locus DNA probes were cloned and used to detect restriction fragment length polymorphisms (RFLP) in lesser snow geese (*Anser caerulescens caerulescens*) (Quinn and White 1987). The conservation of minisatellite "core" sequences was observed to exist in a other species (Jeffreys and Morton 1987) allowing multilocus DNA "fingerprinting" to be used in the study of natural animal populations and behavioural ecology (Burke and Bruford 1987). These developments demonstrated the success of applying DNA markers to address different levels of genetic variability within animal species.

The application of DNA profiling to wildlife forensic science in Canada began in 1989 when conservation officer Nelson Denyes' of the Niagra Region of the Ontario Ministry of Natural Resources requested DNA testing on several samples involved in the suspected poaching of a white-tailed deer. The case required a genetic match between samples collected at an illegal kill site to tissue samples associated with a suspected poacher. In 1989, following Conservation Officer Denyes case, a number of investigations were submitted by the Ontario Ministry of Natural Resources that required testing to determine links between the illegal kills of moose and white-tailed deer to suspected poachers. The hypervariability and cross-species homology of minisatellite

DNA made multilocus DNA fingerprinting the appropriate DNA marker for this question.

DNA evidence obtained in a wildlife forensic investigation made its debut on Febuary 4, 1991 in Sudbury, Ontario. Four individuals were charged under the Game and Fish Act following the illegal shooting of a white-tailed deer in a management unit with a closed hunting season. Three of the individuals were suspected of killing and transporting the animal to a management unit with an open hunting season. A fourth individual was charged for transferring his game seal to the three suspected poachers to generate the appearance of a legal kill. Samples from a gutpile discovered at the site of the original shooting were collected as were tissue samples associated with the suspects and the samples were submitted to Dr. Bradley N. White's laboratory for testing. DNA fingerprinting analysis successfully matched the samples from the illegal kill site to the tissue associated with the suspects. Following Dr. White's testimony, Judge W.G. Mahaffy issue five convictions to the four men: unlawful transportation of game; knowingly possessing illegal game; knowingly possessing illegal game and illegally using a game seal issued to another; and illegally transferring a seal to another. These guilty convictions resulted primarily from the results obtained with the DNA analysis. This precedent setting case demonstrated the court's acceptance of this technology as an effective enforcement tool in wildlife forensic science. In rendering his decision, Judge W.G. Mahaffy expressed no doubt regarding the testimony of Dr. White stating:

"To conclude otherwise in the face of the impressive, scientific evidence which I find most reliable would be an adventure in fantasy." (McLaren 1996).

Since 1989 and the success of the early wildlife investigations submitted for testing, the number of wildlife investigations increased on an annual basis. In 1990-1991 Dr. White's laboratory received funding from the Ontario Renewable Resources Research Program (ORRRG) to develop protocols and process submitted cases. Since 1994 the funding for the DNA research has been provided by the Compliance Operations Section of the Provincial Operations Branch of the Ontario Ministry of Natural Resources (OMNR). In addition to OMNR, the departments of natural resources for each province have utilized the laboratory as well as agencies at the federal level including Parks Canada, Canadian Wildlife Service and the RCMP. The forensic laboratory has been recognized by the federally funded Canadian Cooperative Wildlife Health Centre which has listed the McMaster laboratory in an inventory of Canadian wildlife expertise under the speciality of molecular genetics for wildlife forensic science and genetic stock assessment.

DNA PROFILING

The issues in wildlife forensic science are often more complex than human forensic science because many species rather than one are involved and multiple types of DNA profiling are required. This has been demonstrated since 1989 as the increased number of wildlife investigations included requests to provide evidence in cases that required

different DNA marker systems be established. The development of DNA marker systems for forensic science is based on several principles: 1. the stablity of DNA as a molecule; 2. the sensitivity of DNA analyses to analyse minute sources of biological material; 3. the genetic continuity of DNA, e.g. identical genotypes among tissues; and 4. the ability of DNA profiles to discriminate at different levels, e.g. between species and between individuals. These were the principles we utilized in the development of our DNA profiling protocols.

The cases referred to the laboratory by OMNR and other government agencies have predominantly been of two types: a) Individual identification which has used DNA fingerprinting for matching tissue samples from illegal kill sites to samples associated with a suspect (Guglich et al. 1993, Chapter 1). These poaching cases have largely involved moose (*Alces alces*), white-tailed deer (*Odocoileus virginianus*) and to a lesser degree elk (*Cervus elaphus canadensis*), mule deer (*Odocoileus hemionus*), caribou (*Rangifer tarandus*), wild turkey (*Meleagnis gallopavo*) and even the illegal shooting of a rainbow trout (*Oncorhynchus mykiss*); b) Species identification of unknown tissue samples using highly repetitive DNA markers (Guglich et al. 1994, Chapter 2) has been required in cases involving illegal commercialization and poaching. The species which have been identified include those mentioned above and black bear (*Ursus americanus*), coyote (*Canis latrans*) and Canada goose (*Branta canadensis*). Additional requests from conservation officers for the analysis of specific cases have required modification of

existing methodology or the development of new DNA marker systems to address these needs. These marker systems include: c) Identification and quantification of game animal tissue mixed with domestic animal tissue, i.e. sausages and processed meat products, for the enforcement of illegal commercialization. We modified our existing species identification protocol (Guglich *et al.* 1994) to detect and quantify game tissue in mixed sources at levels of less than 1% game tissue in commercial meats (Chapter 3); d) Sex identification of moose, white-tailed deer and elk in samples lacking gender-specific physical characteristics to enforce the violations of the game validation tag system. The protocol utilizes the polymerase chain reaction (PCR) to simultaneously amplify a region of Y-chromosome gene and a region of a gene on the X-chromosome (Chapter 4); and e) Parentage analysis using DNA fingerprinting to determine if captive breeding programs are introducing wild stocks and claiming the animals are the offspring of registered breeding pairs.

The developments listed above have focused on the specific needs of conservation officers and more recently the research has focused on the problem of poor quality and/ or low yields of DNA in forensic samples caused by extended periods of post-mortem decay (Bar et al. 1989, Ludes et al. 1993) or environmental insults (McNally 1989). Between 10-20% of the cases submitted for testing involve samples of this nature which have prevented a successful analysis. These samples set limits on the aforementioned principles, specifically stability and sensitivity, and are not conducive to the RFLP

analysis standardly used for individual identification and species identification and require alternate DNA marker systems. Applying PCR approaches to such samples allows the analysis of extremely low concentrations and partially degraded DNA samples. We have applied DNA amplification and sequencing of a region of the mitochondrial gene cytochrome b to the species identification of unknown samples. Two recently completed projects have demonstrated the applicability of this type of DNA profile. The first study was funded by the Southern Science, Technology and Transfer Unit of OMNR to establish species-specific markers in fish and wildlife species (Chapter 5). The second project analyzed samples purchased as seal penises in North American and Asian cities (Chapter 6). Sequences obtained from control samples and EMBL/Genbank were used in a phylogenetic approach to determine the species origin. The majority of penis samples were identified to the species of seal and the remaining samples were excluded as seal penises and originating from species which presently lack the appropriate sequence data bases to identify them to the species level.

CHAPTER ONE

APPLICATION OF DNA FINGERPRINTING TO ENFORCEMENT OF HUNTING REGULATIONS IN ONTARIO

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Abstract: DNA fingerprinting has been used in investigations of forty 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 Jeffreys's 33.15 multilocus probe were used to link the animal remains found at the illegal killsite 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 probablility of two individuals sharing the same DNA fingerprint. Details are presented cases in which the evidence was presented and accepted by Ontario courts.

KEYWORDS: DNA fingerprinting, white-tailed deer, moose, poaching

Introduction

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 killsite of an illegally hunted animal by matching blood or tissue from the dead animal at the killsite 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 databases 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 non-inbred populations have been found in the range of 0.2-0.3. Factors which increase the average level of band-sharing, such as population structure [*9*] and linkage among DNA fragments [*10*], must be examined. 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 forty 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 identitical DNA fingerprints from two unrelated animals. Here, we present the application of this technique to 4 cases.

Materials and Methods

DNA Extraction

Tissue samples (0.5 - 0.8 g) were ground in liquid nitrogen with 3.5 milliliters (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 isopropanol. Precipitates were centrifuged at 7,000xg for 30 min, washed with 70% ethanol and recentrifuged. The DNA was dissolved in 250 - 500

microlitres (µl) of 1 X TNE_2 (10 mM Tris-HCl pH 8.0, 100 mM sodium chloride (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 micrograms (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 mg/ml) at 37°C for 1 hour. Each digestion was extracted and precipitated as previously described and dissolved in 40 ml 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 minutes. 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 24-26 hours. Five nanograms (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 X SSC (10 X 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 hours. We used three probes in our analyses, the Jeffreys's 33.15 minisatellite probe [*1*], 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 x 10^9 cpm/µg. Hybridization was carried out at 65°C for 20 - 22 hours. Blots were washed in 2 x SSC, 0.1% SDS; once at room temperature for 15 minutes, and twice at 65°C, for 15 minutes then 30 minutes. After rinsing in 1 x SSC, blots were exposed to Cronex 4 or Kodak XAR X-ray film using one intensifying screen at -70°C for 7 days. Southern blots were stripped between hybridizations in 0.4 N sodium hydroxide at 42°C for 30 minutes, then neutralized in 0.1 x SSC, 0.5% SDS, 0.2 M Tris-HCl (pH 7.5) for 30 minutes 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 3 times and the mean from these 3 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 - 20 kb, 5 - 10 kb, and 2.5 - 5 kb (white-tailed deer) or 2.1 - 5 kb (moose) were used, whereas in Galbraith et al. [*16*] the number of 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 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-tailed 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 siezed from the suspect and that obtained from blood found in a plastic bag found near the probable kill site. Digestion of both samples with *HaeIII* 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. Fourteen white-tailed deer from Manitoulin Island were digested with *HaeIII* and probed with Jeffereys' 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 coefficients for pairs of individuals ranged from 0.19 - 0.84 (mean= 0.47) for the

FIGURE 1. Autoradiograph of *Hae*III digested DNA probed with the *per* locus 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 which corresponds to the band seen in lane 1.

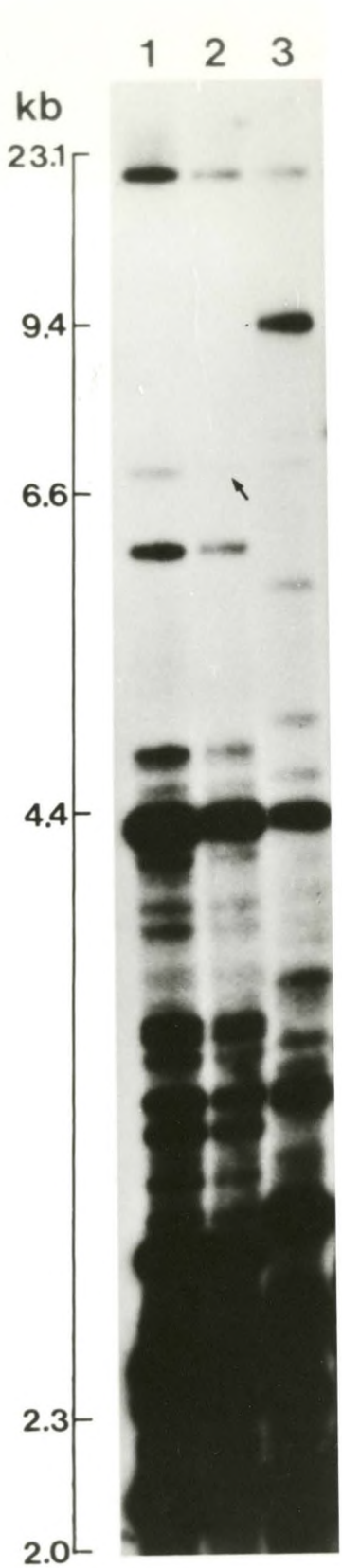
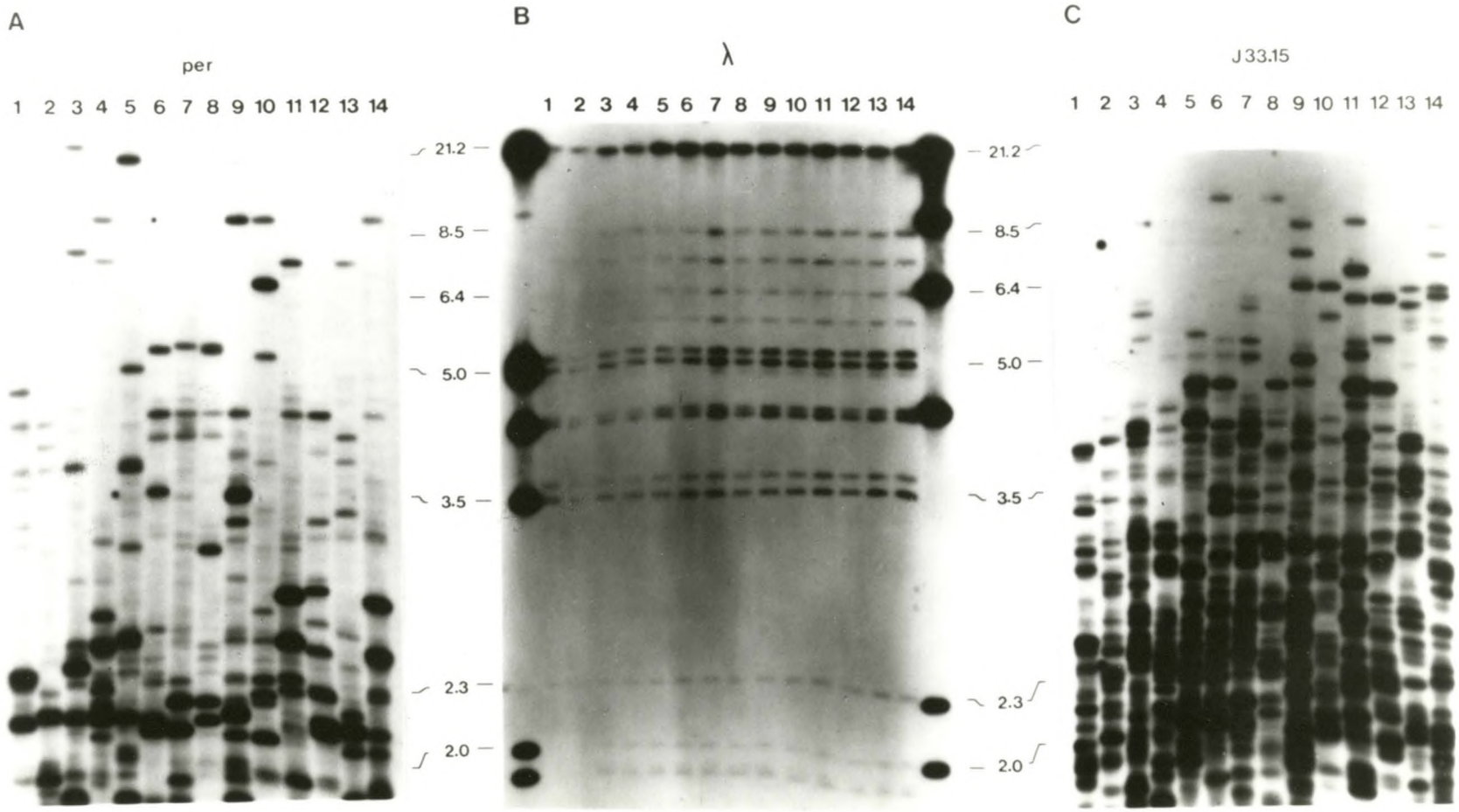

1
2
3
kb
23.1
9.4
6.6
4.4
2.3
2.0

FIGURE 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 throught a 0.8% agarose gel, blotted, and hybridized with the indicated probe. Specific activities were 1.0-2.0 x 10^9 cpm/μg.


A
per
1 2 3 4 5 6 7 8 9 10 11 12 13 14
21.2
8.5
6.4
5.0
3.5
2.3
2.0
B
λ
1 2 3 4 5 6 7 8 9 10 11 12 13 14
21.2
8.5
6.4
5.0
3.5
2.3
2.0
C
J33.15
1 2 3 4 5 6 7 8 9 10 11 12 13 14

per probe. To estimate the likelihood of two animals sharing the same DNA fingerprint a higher band-sharing coefficient for the *HaeIII/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 indentified in the buck's DNA fingerprint was therefore estimated at $(0.60)^{22}$ or 1.31×10^{-5}. Therefore the likelihood that the blood in the plastic bag came from the buck's head found in the possession of the suspect's was estimated as greater than 100,000:1.

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 establish a mean band-sharing coefficient for the 33.15/*HaeIII* combination from animals across the province (Table 1). For the 27 animals analysed 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 forund 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

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 [17].

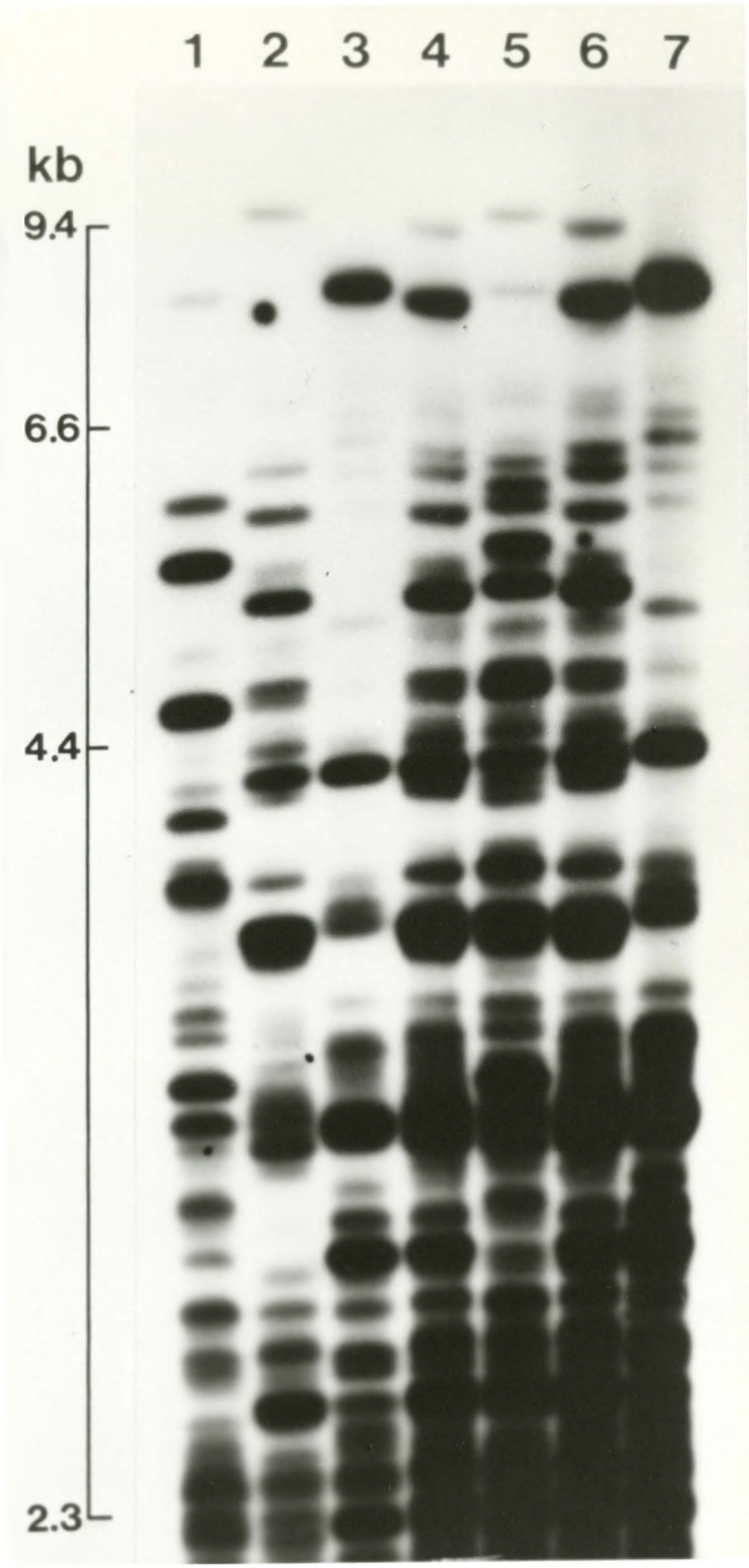
[b] A larger band size range was examined in moose to obtain a greater number of informative DNA fingerprint bands.

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 4-6) had a greater number of bands than those from tissues at the kill sites (lanes 1-3). 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) ot 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 *AluI* and *HaeIII* and hybridized with both the *per* and Jeffreys's 33.15 probes (Fig.4). The *AluI* Jeffreys's 33.15 derived fingerprints gave the 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

FIGURE 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 2 bucks (2 and 3), and 4 different preparations from the suspect's blood-stained snowmobile suit (4-7).

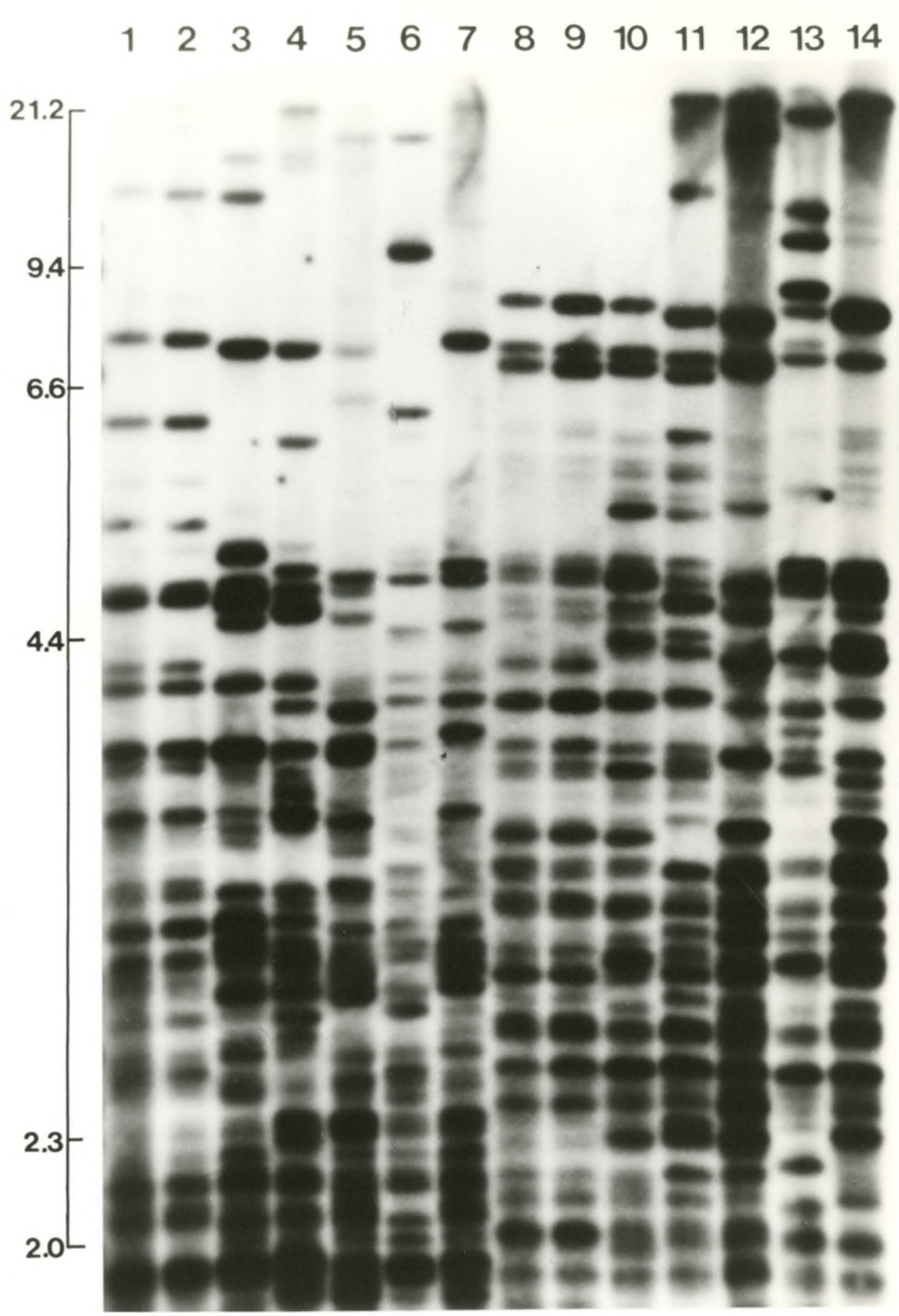

1 2 3 4 5 6 7
kb
9.4
6.6
4.4
2.3

33.15/*HaeIII* (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 this 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 *AluI* and *HaeIII* digested DNA probed with Jeffrey's 33.15, all of the bands from the tissue at the kill site matched those derived from the meat in the freezer. The number of bands scored in the *AluI* digest was 22 and therefore the probability that these fingerprints were derived from 2 different animals was estimated as $(0.6)^{22}$ or 1.31×10^{-5}.

The second case involved a group of hunters suspected of poaching 5 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 2 liver samples obtained from the supsect'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 (lane 2) migrated faster than those from the liver (lanes 3 and 4). When these were

FIGURE 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 5 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.


1
2
3
4
5
6
7
8
9
10
11
12
13
14
21.2
9.4
6.6
4.4
2.3
2.0

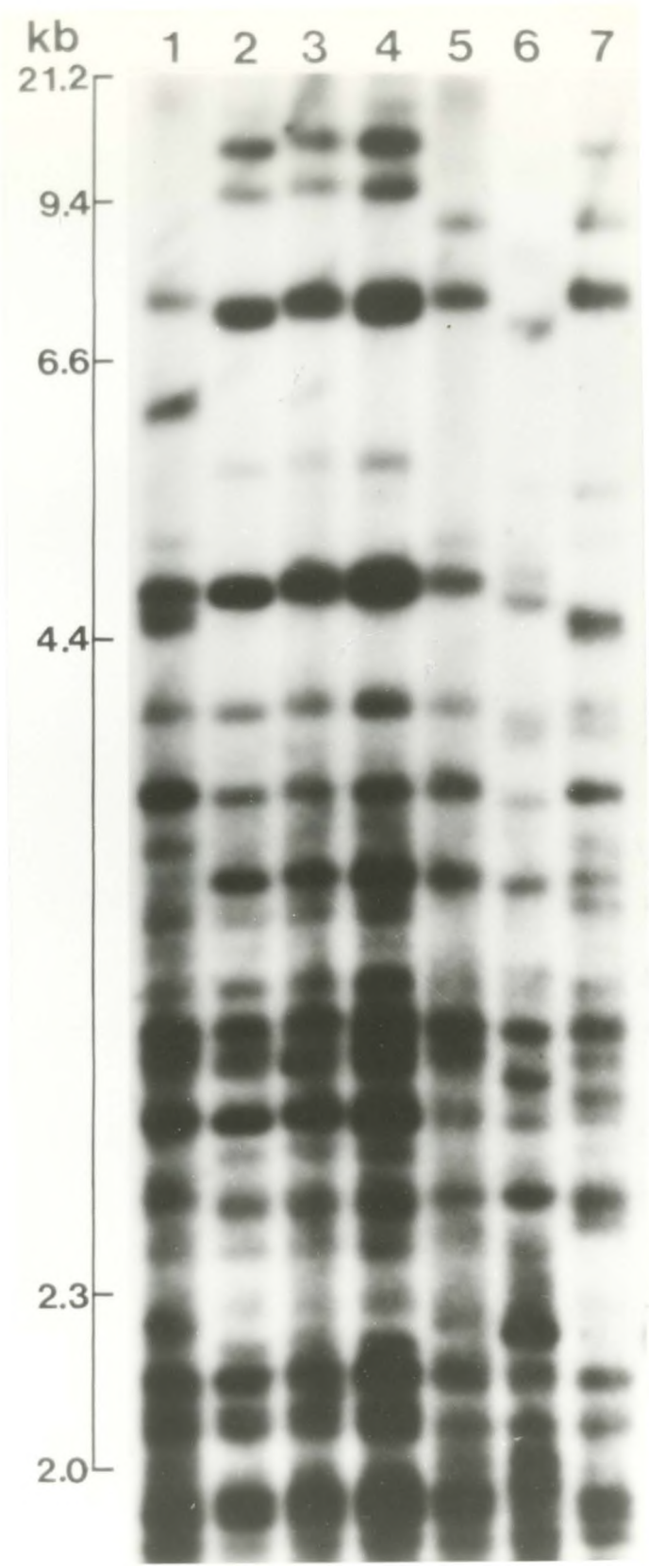
assessed using the internal lambda markers the sizes of all 23 bands matched. The probability that the liver came from an animal other than the yearling bull moose found at the kill site was estimated as $(0.60)^{23}$ or 7.89×10^{-6}. The DNA fingerprints obtained from the tissues found in the vehicle and trailer (lanes 5-7) represented 3 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 killsite. 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 (eg. 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

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


kb
1
2
3
4
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21.2
9.4
6.6
4.4
2.3
2.0

the fingerprint. The D values we used in these estimates were modified from the maen 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 byusing 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 favours 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 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 *HaeIII*/Jeffreys' 33.15 combination for white-tailed deer and the *AluI*/Jeffreys' 33.15 combination for moose for the respective DNA fingerprints. Using *HaeIII*/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 *AluI*/33.15. With local populations having a slightly higher value, we used a band-sharing coefficient to 0.60 of 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 [*21*] and protein marker studies [*22*] 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 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 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 turn-over rate for results is 3-4 weeks. We recommend that sample

preparation in the field requires freezing the blood or tissue as soon as possible, or to place the material in preparation kits containing lysis buffer (4 M urea, 0.2 M sodium chloride, 0.5% n-lauroyl sarcosine, 10mM CDTA, 100 mM Tris-HCl pH 8.0; Applied Biosystems Inc.) or pickling solution. The DNA in tissues preserved in these solutions is stable for months at ambient temperature and can be easily transported to the forensic laboratory.

Acknowledgements

We thank A. J. Jeffreys for permission to use the 33.15 minisatellite probe and T. A. Bargiello for providing our lab with the *per* locus probe. We thank D. Harnish, S. Rosa, M. Bailie, M. Jackson, G. Couillard, R. Dupuis, P. Gray, P. Draycott, B. Koomans, M. Buckner, R. Tremblay and others from the Ontario Ministry of Natural Resources for providing tissue samples and cases to investigate. This research was supported by grants from the National Science and Engineering Research Council of Canada and an Ontario Ministry of Natural Resources Ontario Renewable Resources Research Grant to B. N. White.

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CHAPTER TWO

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: species identification, illegal commercialization, white-tailed deer, moose, black bear, repetitive DNA

Introduction

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, 2, 3, 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 which are not well preserved [*4*]. Techniques involving DNA analysis have recently been developed to provide evidence in wildlife forensic cases [*5, 6, 7, 8, 9, 10, 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, i.e. DNA fingerprinting [*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 [*9*]. Mitchondrial DNA markers have been used for species identification [*7*]. The polymerase chain reaction (PCR) has been used for species identification by amplifying rDNA [*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 utilizes repetitive satellite DNA sequences. The high copy number of satelitte 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 which 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 - 0.8 g) were ground in 3.5 ml 1 X 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 7,000 g for 20 - 30 minutes, washed with 70% ethanol. The resultant pellets were dissolved in 250 - 500 ml 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 - 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/ml) at 37 °C for 1 hr. 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 - 1.4%, depending on the fragment sizes to be resolved, at 60 V for 3 - 6 hr in Tris acetate (40 mM Tris, 5mM 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, *Hinf*I, *Msp*I, *Pst*I, *Pvu*II, *Taq*I and *Xho*I. Digestion with *Ava*I, *BamH*I, *Cla*I, *EcoR*I, *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, *Hinf*I, *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,

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

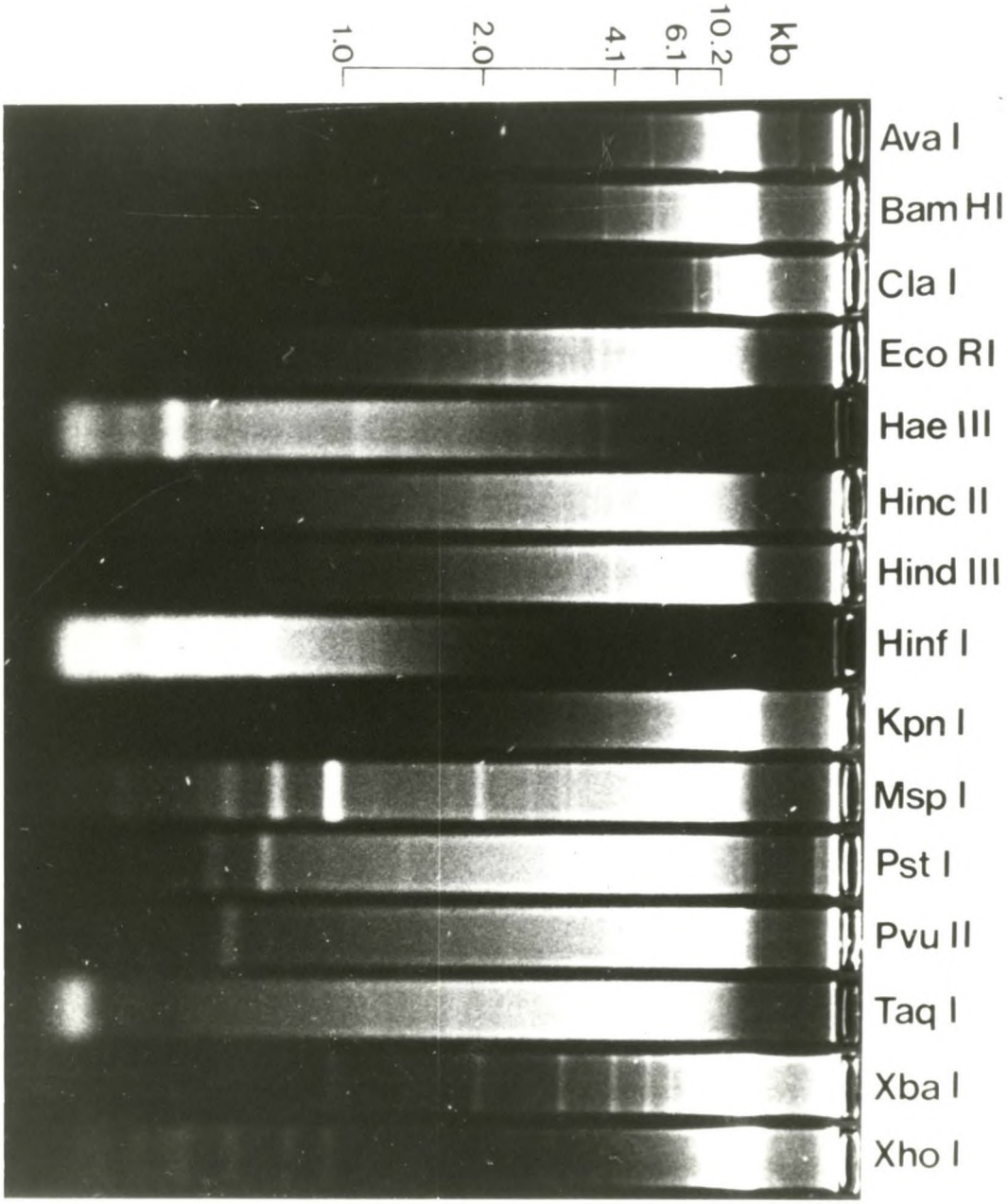
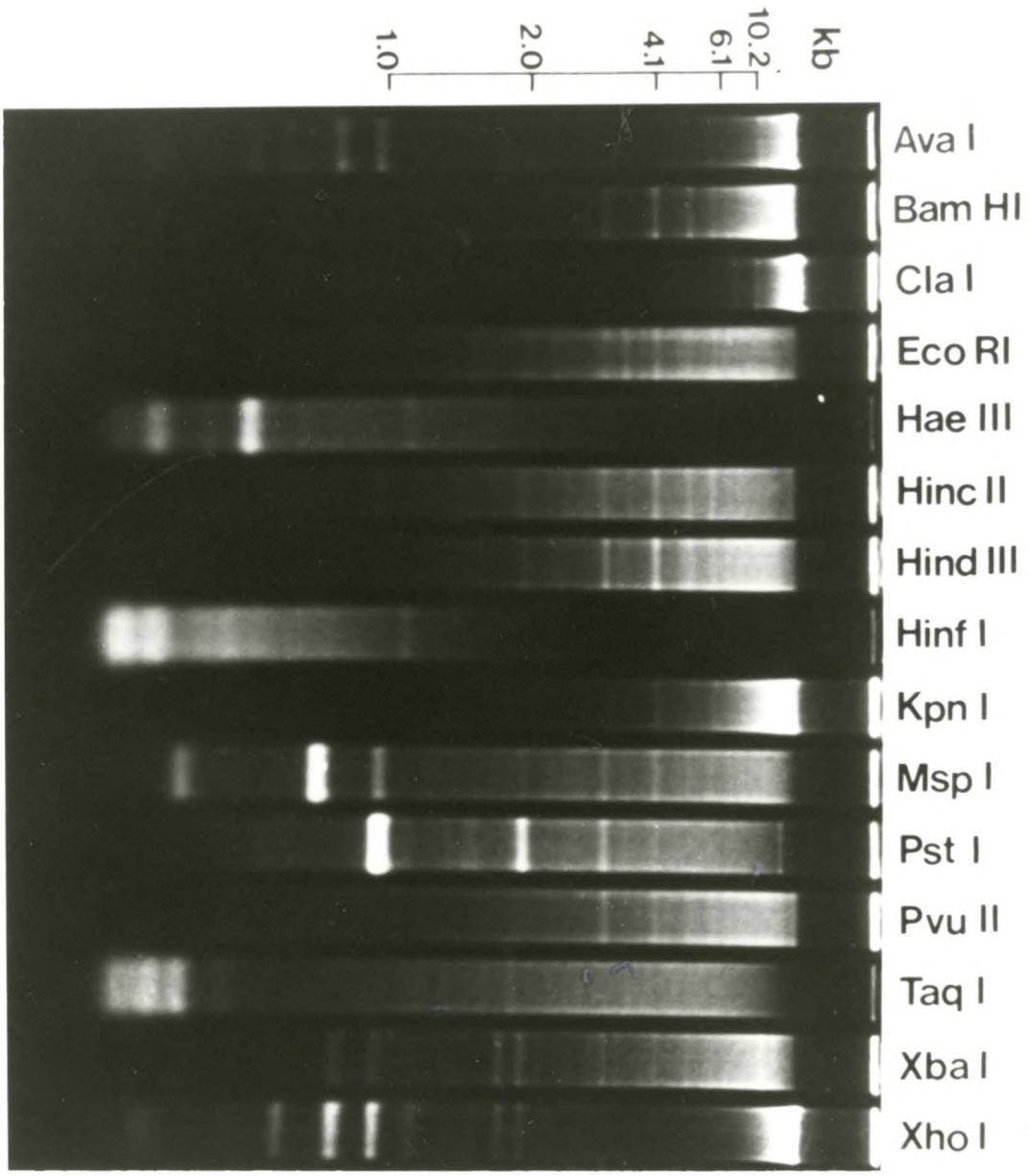

kb
10.2
6.1
4.1
2.0
1.0
Ava I
Bam HI
Cla I
Eco RI
Hae III
Hinc II
Hind III
Hinf I
Kpn I
Msp I
Pst I
Pvu II
Taq I
Xba I
Xho I

FIGURE 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).


kb
10.2
6.1
4.1
2.0
1.0
Ava I
Bam HI
Cla I
Eco RI
Hae III
Hinc II
Hind III
Hinf I
Kpn I
Msp I
Pst I
Pvu II
Taq I
Xba I
Xho I

additional repetitive bands in *Pst*I digested moose DNA distinguished these two species, as did digestion with other restriction enzymes, i.e. *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 proof of the species.

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, i.e. *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 (Aal). The banding patterns of *Xho*I confirmed these results.

FIGURE 3. Ethidium bromide stained agarose gel (1.0%) of DNA (2 µg/lane) digested with *Pst*I and *Xho*I 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).

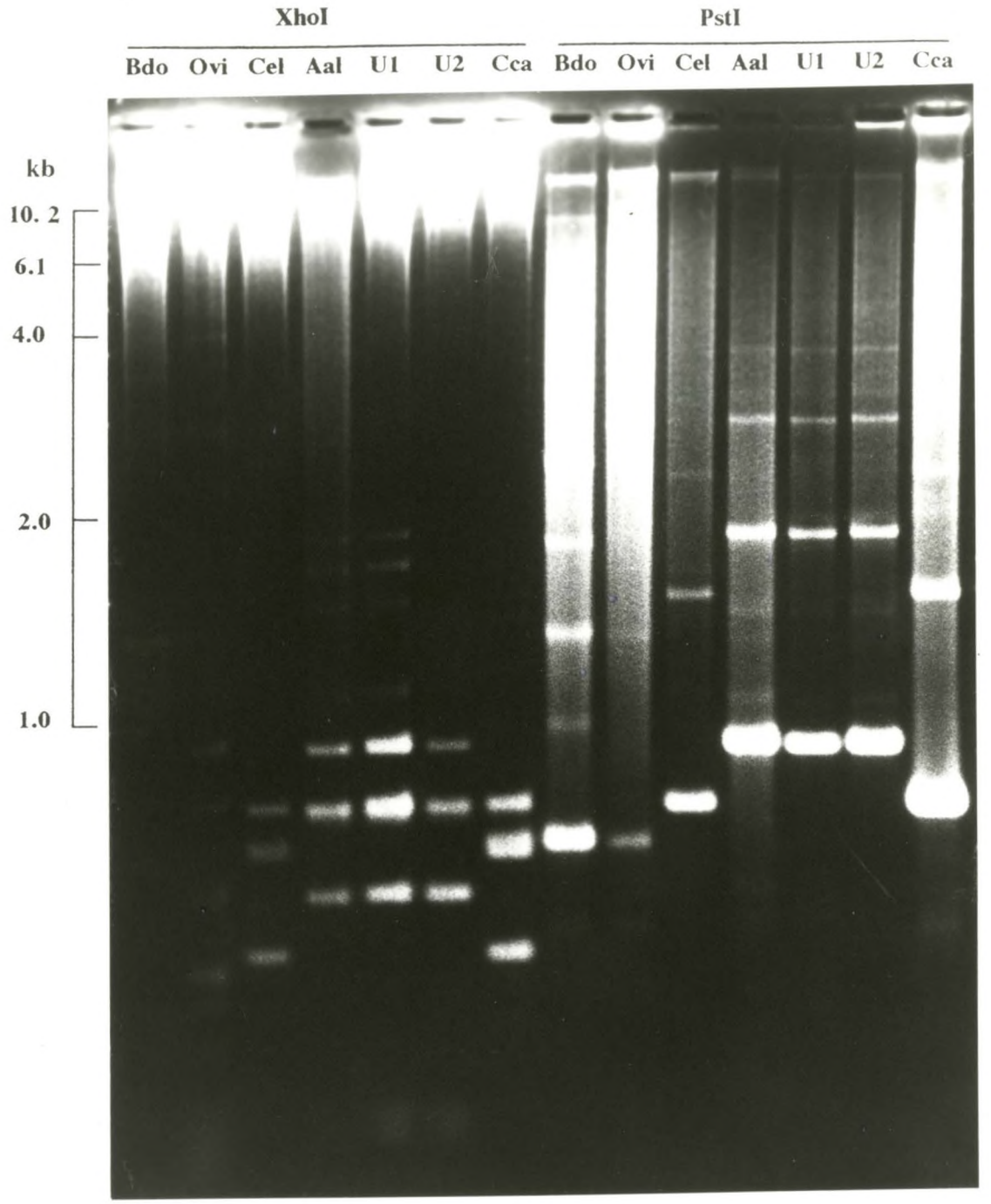
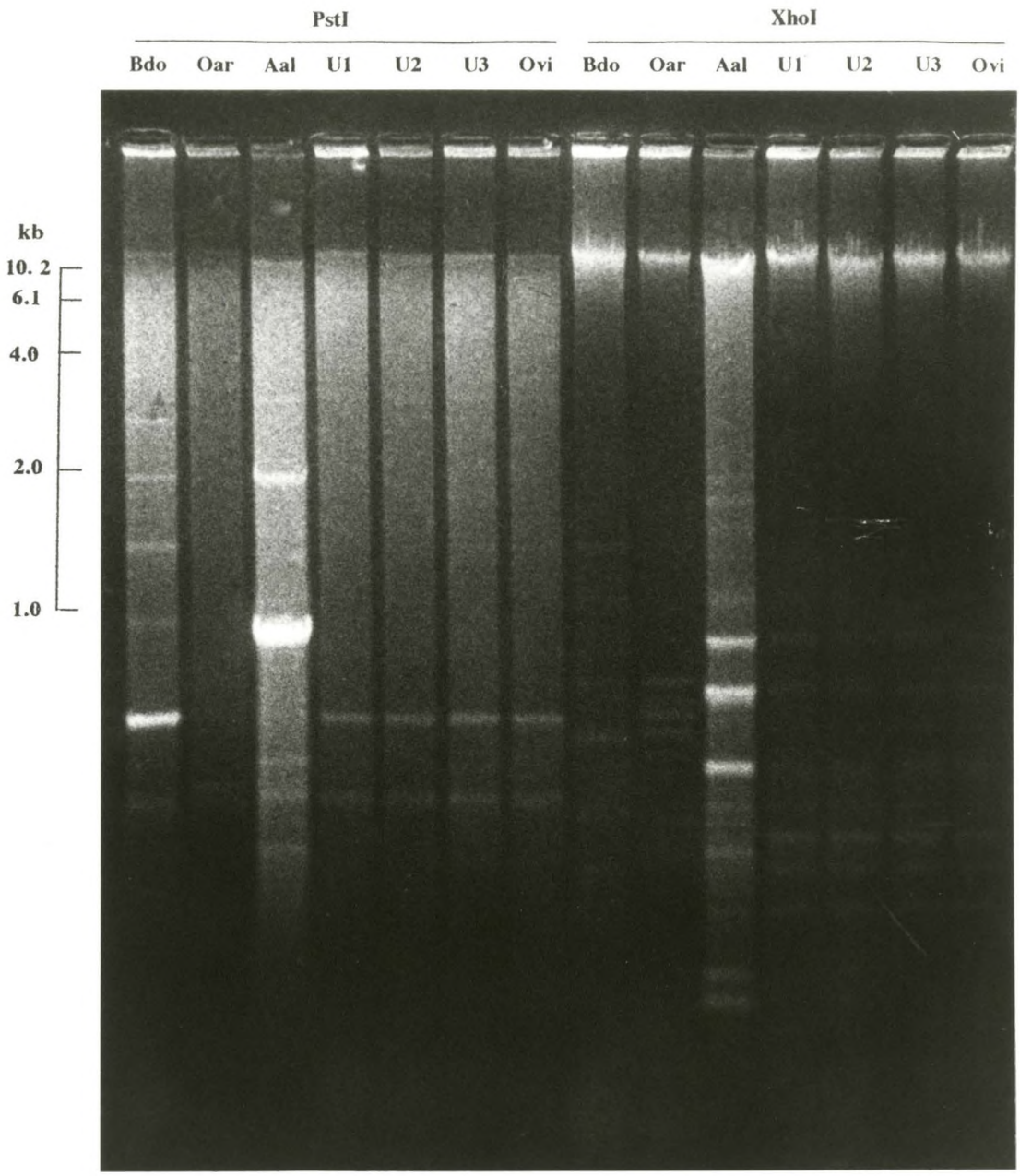

XhoI
PstI
Bdo Ovi Cel Aal U1 U2 Cca Bdo Ovi Cel Aal U1 U2 Cca
kb
10. 2
6.1
4.0
2.0
1.0

FIGURE 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).


PstI
XhoI
Bdo
Oar
Aal
U1
U2
U3
Ovi
Bdo
Oar
Aal
U1
U2
U3
Ovi
kb
10. 2
6.1
4.0
2.0
1.0

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 (Aal) 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.

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, i.e. 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 (Fig. 3 and 4), but the *Pst*I digestion of white-tailed deer, beef and lamb revealed similar banding patterns with only an additional

2 bands to distinguish the three species (Fig. 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 which yield poor quality DNA are not precluded from this type of analysis. Restriction enzymes which 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 (Fig. 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 been 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 intra-

specific 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, 13, 14, 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 intra-specific homogeneity and inter-specific heterogeneity which is ideal for species identification.

Restriction enzyme analysis of repetitive DNA examines species-diagnostic sequence variants, i.e. 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, i.e. 15 restriction enzymes, should be used to compare the species of interest to other species associated in the context of common violations, i.e. a game animal species compared to various domestic species. Restriction enzymes can be identifed which reveal prominent banding configurations, demonstrate inter-specific 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 10-20 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 utilize radioactive molecular hybridization to identifiy 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*]. Intra-specific 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 utilizing DNA analysis. The analysis of extracted DNA using diagnostic restriction enzymes can produce results in less than 8 hours. Analyses requiring probe labelling, i.e. mtDNA analysis [*7*], hybridization with isolated repetitive DNA [6] and

sequencing [*8*], require 3-7 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 labelling is higher than the cost per sample of analyzing visible repetitive bands.

One disadvatage 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, i.e. blood stained clothes and knives, is in the range of 5-10 ug which is sufficient for analysis. Smaller amounts can be further analyzed by Southern blotting and applying one of the techniques utilizing DNA probes, i.e. mtDNA probes [*7*] or repetitive DNA probes [*6, 9*] to increase sensitivity. Another disadvatage 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 repetitve DNA markers for species

identification we have presented in Ontario provincial court have been accepted as evidence.

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CHAPTER THREE

IDENTIFICATION AND QUANTIFICATION OF GAME TISSUE IN PROCESSED MEAT PRODUCTS

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Abstract: A number of molecular techniques have been developed to identify the species of origin of unknown tissue samples in wildlife forensic cases. Illegal commercialization investigations may require the detection of game meat that has been mixed with domestic meats. We have modified an existing protocol using highly repetitive satellite DNA markers to identify and subsequently quantify the amount of game tissue present in a processed meat sample. ^{32}P-radioactively labeled genomic DNA of the game species being detected is hybridized to Southern blotted DNA digested with species diagnostic restriction enzymes and, following highly stringent washing conditions, an autoradiograph reveals species-specific banding patterns with essentially no cross-homology to other game or domestic species. Comparing signal intensities of satellite DNA bands between case and control samples of known concentration allows a relative quantification of the game tissue present in a processed meat sample. Data are presented from an Ontario Ministry of Natural Resources case that demonstrates the effectivness of this technique.

KEYWORDS: illegal commercialization, wildlife forensic science, moose, white-tailed deer, meat products

Introduction

The application of DNA marker systems to the species identification of an unknown tissue sample has been well established for wildlife forensic science using a number of techniques. The molecular systems used in species identification include: restriction fragment length polymorphisms (RFLP) of highy repetitive satellite DNA [*1, 2, 3, 4*]; and the amplification of regions of mitochondrial DNA[*5*] and nuclear rDNA [*6*] using the polymerase chain reaction (PCR). All the above methods are effective in the identification of a unknown meat sample associated with a poaching infraction or an investigation involving the illegal commercialization of game meat. A second type of illegal commercialization infraction involves the mixing of game meat with domestic meat in processed meat samples, e.g. sausages. In these infractions a butcher is typically suspected of substituting inexpensive game meat into meat products where the suspect sells the products illegally to knowing buyers or the buyer is unaware of the presence of game meat. Infractions of this nature are often in violation of both Fish and Game Acts as well as Food and Agriculture legislation.

All the species identification molecular techniques that have been developed can identify the presence of game animal within a mixed source, however, techniques utilizing PCR technology to amplify the species-specific marker are limited. The illegal commercialization of mixed meat products requires a determination of the amount of game animal that is present which can not be assessed using the highly sensitive amplification

of DNA. As these violations are most often associated with butcher shops, the presence of game animal is not grounds for proving illegal commercialization because butchers can legally cut and package harvested game animal for hunters using the same meat processing equipment. The identification of game animal in a processed meat sample at very low levels may only reflect the contamination of tissue derived from game animals indicating a potential hygene issue but not the intentional addition of game meat for the purpose of sale. This limitation to PCR based techniques favors the RFLP analysis of highly repetitive satellite DNA and the potential application of satellite DNA probes for this type of case have been addressed [*1, 2, 4*]. We have modified an existing protocol using highly repetitive DNA [*4*] to generate a rapid test for identifying and quantifying the presence and amount of game tissue present in a mixed processed meat sample. This application will identify and quantify illegal meat sources within commercial products with minimal research and development as a result of eliminating the cloning of specific satellite DNA repeat units.

Materials and Methods

DNA Extraction

Samples were frozen prior to analysis to preserve the DNA. Processed meat samples were homogenized with a Sorvall Omni Mixer. A portion of the each tissue sample (0.5 g) was ground in liquid nitrogen with 3.5 mL 1X 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.). Samples were incubated at 37°C for up to one week. Proteinase K (62.5 U; Applied Biosystems Inc.) was added to each sample and was incubated at 37°C for 12 hours, followed by a second incubation with proteinase K at 37°C for an additional 12 hours. 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 10 M ammonium acetate and 2X volume of 95% ethanol. Precipitates were centrifuged at 3000 x g for 15 minutes, washed with 70% ethanol and recentrifuged. The DNA was dissolved in 1x TNE_2 (10 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, 2 mM EDTA (disodium ethylene diamine tetraacetate•2 H_2O). The amount of DNA in a sample was quantified using a TK 100 mini-fluorometer using a dye (Hoechst 33258).

Restriction Enzyme Analysis

DNA (1-2 μg) was digested with restriction enzymes (5 U/ug) as described by the manufacturer (BRL). Agarose gel electrophoresis was used to separate the DNA fragments following amplification or restriction enzyme digestion. DNA was electrophoresed through agarose gels using an electrical current and submersion in buffer (TBE). Flanking molecular weight size standards of 0.2 μg/lane of bacteriophage lambda DNA digested with *HindIII*, were run on each agarose gel. An aliquot of the volume of

the reactions plus one-fifth volume of gel loading buffer (0.5% orange g, 15% ficoll, 50 mM EDTA, pH 8.0) were run on agarose gels. DNA was electrophoresed through 0.8% to 1.2% agarose gels at 60V for approximately 12 hours in Tris borate buffer (89 mM Tris, 89 mM boric acid, 2mM EDTA, pH 8.0). Agarose gels were stained with ethidium bromide (final concentration 2 μg/ml) for 30 minutes and were visualized with ultraviolet (U.V.) light.

Southern Blot Analysis

The digested DNA was transfered to a positively charged polyvinylidene difluoride membrane (Immobilon-N, Millipore Corporation) according to the manufacturer's protocol and the method described by Southern (1975). The membranes were prehybridized for 12-20 hours at 65°C in a solution of 7% SDS (sodium dodecyl sulfate), 1 mM EDTA (pH 8.0), 1% bovine serum albumin, 0.25 M sodium ortho-phosphate (pH 7.2). Genomic DNA (25 ng) of moose or white-tailed deer was radioactively-labeled by random primer extension (Feinburg and Vogelstein 1983) with [alpha^{32}P] dCTP. Membranes were hybridized with the radioactively-labeled genomic DNA for 12-20 hours at 65°C and then washed with 0.1X SSC (10X SSC: 1.5 M NaCl, 0.15 M sodium citrate), 0.5% SDS at 70°C for 15 minutes, 30 minutes and 20 minutes. Membranes were first exposed to a Phosphoimager Screen (Kodak) followed by exposure

for 1-2 days at -70°C to Cronex X-ray film (Dupont) and Lighting Plus Cronex intensifying screens (Dupont).

Quantification of Repetitive DNA Markers

Highly repetitive DNA markers were quantified using the ImageQuant (Molecular Dynamics) software. Radioactive Southern blots were exposed to a Phosphoimager Screen (Kodak) and scanned by a Series 400 Phosphoimager (Molecular Dynamics). The Quantitation component of the ImageQuant software provided a measurement of the intensity of individual repeat bands using the Define Background and Integrate Volume options. One background level per blot was used for integrating volumes as the banding patterns demonstrated a dense intensity with minimal backgroud signal. The volumes for each intensity were calculated by the number of pixel values minus the established background. The values were standardized to account for the differences in the amount of DNA: 1 ug of DNA for the case samples and 200 ng of control DNA. The repeat bands of the case samples were compared to the control sample to estimate the percentage difference between the two samples.

Results and Discussion

Repetitive satellite DNA markers have been applied the the species identification of unknown tissue samples in wildlife forensic investigations [*1, 2, 3*] and the species-

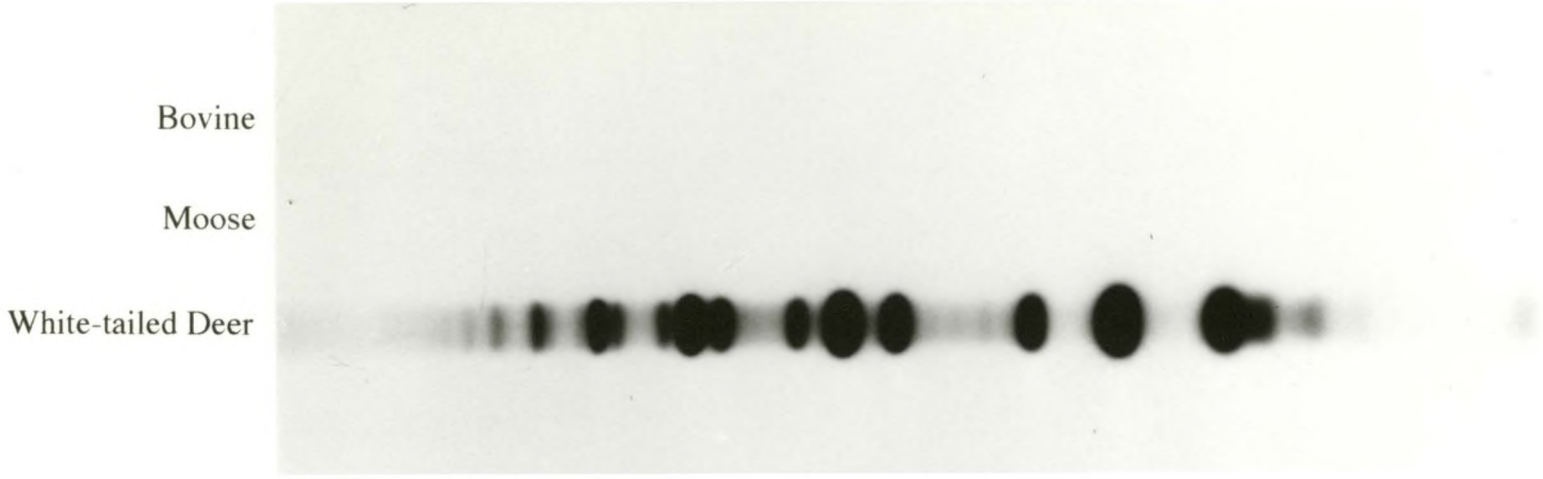
specific nature of satellite DNA is well established. Satellite repeat families in ungulate species evolved from common origins and subsequently have undergone structural and sequence divergence [*9, 10, 11. 12*]. Concerted evolution is proposed to homogenize variant repeat units differing in sequence and size among individuals of a species [*13, 14, 15, 16*]. Structural changes alter the size of the repeat units and these variants are suggested to have evolved from recombination between different repeat elements followed by their amplification into arrays and homogenization through a species [*9, 10, 17*]. A divergence rate of 1×10^{-5} base substitutions per nucleotide site per year has been estimated for one of the artiodactyl repeat families, satellite II [*11*] and this corresponded to approximately 40% sequence divergence between cervid and bovid species. Additional repeat families cloned from other Cervidae demonstrate levels of sequence divergence of 40% between species [*10*]. The intra-specific variation among repeat units observed in the satellite II repeat family of white-tailed deer was estimated at approximately 2.5% [*11*] and low levels of intra-specific sequence divergence have been observed among the monomer untis of repeat families in other species [*16*].

The high levels of inter-specific sequence divergence and differences in the size of repeat units among species may correspond to species diagnostic differences in restriction enzyme perodicities and the size of repetitive DNA bands visualized following gel electrophoresis or Southern blotting analysis. The low intra-specific and high inter-specific sequence variation results in species-specific hybridization under highly stringent

conditions [*1*]. Another characteristic of satellite DNA is the high copy number of repeat arrays typically representing a percentage of a species genome: e.g. the Rt-Pst3 repeat units of caribou comprises 5.7% of the genome [*10*]; the PstI repeat of moose contributes 33% of the genome [*18*]; and 1.7% of the satellite II white-tailed deer monomer [*11*]. The copy number of repetitive satellite DNA markers has been utilized in identifying distinct banding patterns in ethidium bromide stained DNA samples following restriction enzyme digestion [*3*]. We have further utilized the high copy number of the repeat units to the identify satellite DNA markers with Southern blot analysis. Hybridization of a sample with ^{32}P-labeled genomic DNA of the species being detected, i.e. radioactive moose genomic DNA hybridized to digested moose DNA, generates repetitive DNA banding patterns at an intensity substantially higher than single copy DNA allowing the visualization of repeat families without using the cloned monomers of the satellite DNA families as probes [*1, 2*].

Figure 1 demonstrates the species-specificity of the hybridization of radioactively labeled genomic DNA, specifically white-tailed deer, to the repetitive DNA markers of that species. The DNA from samples of white-tailed deer, moose and bovine was digested with the restriction enzyme *MspI* and visualized under U.V. light following ethidium bromide staining (data not shown). The repetitive DNA banding patterns for each species were identical to the species-specific *MspI* patterns expected for white-tailed deer and moose [*3*] and the domestic species. Southern blot analysis of the DNA

FIGURE 1. Autoradiograph of *MspI* digested DNA from white-tailed deer, moose and bovine (200 ng/lane). The DNA was hybridized with ^{32}P-radioactively labeled genomic white-tailed deer DNA and washed at high stringency.


Bovine
Moose
White-tailed Deer

hybridized with ^{32}P-labeled genomic white-tailed deer DNA followed by highly stringent wash conditions revealed the *MspI* repetitive DNA patterns of white-tailed deer (Fig. 1). Weak signal was observed in the moose and bovine samples at longer exposures indicating low levels of cross-hybridization with other ungulate species, however, the repetitve DNA banding patterns were consistent with the *MspI* patterns observed for these species thereby eliminating the possibility of a false identification.

We have applied this protocol to actual wildlife investigations. One case from northern Ontario involved a butcher suspected of processing moose meat into his sausage products (Table 1). The sausage products were for sale to private individuals and through local supermarkets where the buyers were unware of the presence of game meat in the sausages. Samples of processed meat, e.g. sausages, were homogenized to provide an representative DNA sample of any of the species present in the product. The sampling of only a section of the meat product may fortitously yield more game animal than the sample as a whole and therefore demonstrate a higher overall content of game species DNA than is actually present. The homogenization of case samples will result in a conservative sampling of the product by preventing an inflation of the amount of non-domestic tissue present in a sample. The term 'conservative' in a forensic context is defined as providing evidence that does not bias against a suspect [*19*], a criteria that is consistent with our sample extraction procedure.

The samples were analysed with the restriction enzyme *PstI* which is the diagnostic restriction enzyme for identifying a sample as originating from moose [*3*]. Upon visualization of the ethidium bromide stained DNA of control samples (moose, bovine and pig), the species-specific repetitive DNA banding patterns for each species were observed (data not shown). Five of the ten samples (K3, K4, K5, K9 and K11) contained the 1kb *PstI* repeat band observed in the moose control sample. The repetitive DNA markers of bovine or pig were present in the case samples and several samples contained banding patterns of both domestic species. The presence of bovine DNA in samples designated as ham sausages (Table 1) further demonstrates the admixture of meat products into processed meats, although this finding would not be under the juristdiction of Fish and Game Acts.

The detection of moose repetitive DNA markers at low percentages prior to Southern blot analysis is due to the extemely high copy number of the *PstI* satellite DNA family in moose. This repeat unit has been estimated to represent 33% of the total genome of moose at a copy number of 1,000,000 [*18*]. Despite the detection of the moose 1kb *PstI* repetitive DNA band with ethidium bromide staining, the additive effect of multiple species within a sample decreased the resolution of the banding patterns such that not all the repetitive bands typical of a species were visible, e.g. dimers and trimers. The multiple partial banding patterns derived from more than one species were therefore not consistent with the control DNA samples. These discrepencies in the detection of

TABLE 1. Samples involved in an investigation involving a butcher suspected of illegally mixing game meat with domestic meat for the purpose of sale. The percentages of game species DNA were calculated by comparing the intensity of moose repetitive DNA bands in the case samples to the control moose sample following Southern blot analysis and hybridization of the *PstI* digested DNA with ^{32}P radioactively labeled genomic moose DNA.

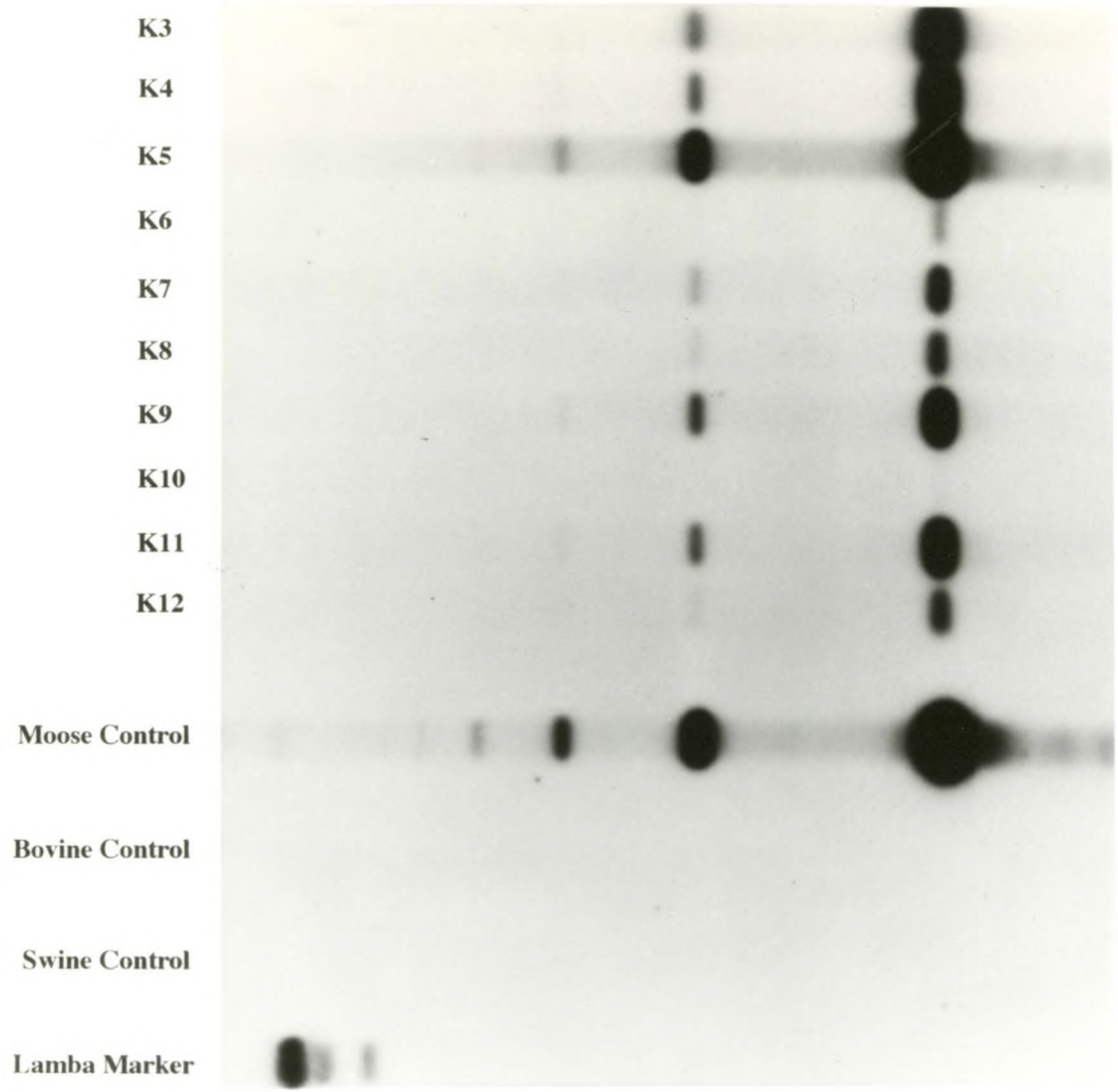
Sample	Type	Percentage of Moose DNA
K3	Smokie Sausage	4.4%
K4	Farmer Sausage	4.7%
K5	Cheese Smokie Sausage	11.0%
K6	Smoked Farmer Sausage	<0.1%
K7	Ham Garlic Ring	<0.1%
K8	Ham Garlic Ring	<0.1%
K9	Ham Garlic Ring	2.6%
K10	Ham Garlic Ring	0.0%
K11	Ham Garlic Ring	2.7%
K12	Smoked Farmer Sausage	<0.1%

moose DNA in mixed samples with ethidium bromide staining indicates this procedure can only be considered a preliminary test. Therefore to provide definitive evidence that the repetitive DNA markers are moose-specific and to quantify the moose DNA present in a sample requires hybridization with labled genomic moose DNA.

Following Southern blot analysis by hybridizing the control and case samples with radioactively labeled genomic moose DNA, the presence of moose was revealed in nine of the ten sausage samples (Fig. 2). Comparing the intensity of the low molecular weight *PstI* monomer unit in the case samples containing moose to the control DNA sample of moose provided an estimate of the amount of game DNA present in each sausage sample (Table 1). The amount of moose DNA present in the sausage samples was between 0.7 % and 11.0% (Table 1). The detection of moose *PstI* repeats was possible in ethidium bromide stained DNA samples containing at least 2% or moose DNA while samples containing less than 1% of moose DNA within the sample did not reveal visible moose-specific repetitive DNA markers. Hybridizing the *MspI* digested DNA of the case samples with radioactively-labled genomic white-tailed deer DNA did not reveal the presence of this species (data not shown).

We selected the monomer satellite DNA units for the quantification of game meat in processed samples because scoring the monomer units will prevent the over-estimation of the intensity of satellite DNA markers in case samples with partial restriction enzyme digestion. A partial digestion will artificially elevate levels of dimer units, trimer units,

FIGURE 2. Autoradiograph of *PstI* digested DNA from control samples of moose and bovine and swine (200 ng/lane) and ten case samples of processed meat products (1μg/lane). The DNA was hybridized with ^{32}P-radioactively labeled genomic moose DNA and washed at high stringency.


K3
K4
K5
K6
K7
K8
K9
K10
K11
K12
Moose Control
Bovine Control
Swine Control
Lamba Marker

ect. and therefore the scoring of these bands would bias the result to elevating the relative intensity in a non-conservative manner. Also DNA degradation may cause the opposite effect in dimer, trimer and larger satellite DNA units by reducing the actual number of repeat units present. Therefore the monomer units represent the most appropriate repetitive DNA marker for quantification. We compared the intensities of the dimers and trimer repeat units among the case and control samples. The values were not proportional to the values obtained from the intensities of the monomer units, for example sample K2 contained 4.4% moose DNA based on the monomer unit and 3.7% when based on the dimer unit. This reduction in intensities between the monomer and the dimer repeat units was observed in several samples and is likely the result of DNA degradation in the meat products that reduced the number of the larger repeat units. Low levels of DNA degradation in processed meats may result from procedures such as smoking or other treatments.

In the above investigation the results were presented in terms of the percentage of game DNA present in each case sample. These percentages provided a conservative estimate of the proportional amount of game tissue present in the processed meat samples. Several of the samples were at sufficiently high percentages to discount the possibility of contamination from uncleaned butchering equipment and the suspect, upon reading the DNA report following disclosure, altered his plea to guilty on one count of illegally selling moose and one count under the Ontario Agriculture and Food legislation.

The Ontario provincial court has since admitted this technique into evidence in subsequent investigations, thereby establishing precedence.

We have modified an existing protocol for identifying the species of origin in unknown tissue samples [*3*] to accurately detect and quantify the amount of game tissue mixed with domestic species in processed meat products. Hybridizing with genomic DNA to visualize species-specific highly repetitive DNA banding patterns eliminates the need to isolate and clone satellite DNA units and this approach can be expanded to a large number of species. Also this protocol is not precluded from differentiating between closely related species that cross-hybridize with the labeled genomic DNA of the species being detected, even under highly stringent conditions, providing species diagnostic restriction enzymes can be established. The one disadvantage of this technique is the requirement for relatively high molecular weight DNA and samples with lower quality DNA may require a PCR-based species identification protocol to identify the presence of game tissue, although the ability to quantify the amount of game meat is limited.

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CHAPTER FOUR

SEX IDENTIFICATION OF ELK (*Cervus elaphus canadensis*), MOOSE (*Alces alces*) AND WHITE-TAILED DEER (*Odocoileus virginianus*) USING THE POLYMERASE CHAIN REACTION

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Abstract: We have developed a PCR based protocol to determine the gender of tissue samples originating from elk (*Cervus elephus canadensis*), moose (*Alces alces*) and white-tailed deer (*Odocoileus virginianus*). The techinique simultaneously amplifies a conserved region of the sex-determining gene on the Y-chromosome (Sry) and a region of the Fragile X mental retardation gene (Fmr-1). The multiplex nature of this protocol allows the determination of gender using the Sry marker with the Fmr-1 marker providing an internal control. This technique is applicable to the enforcement of the validation tag system for game species. Data are provided from a wildlife investigation in Ontario.

KEYWORDS: polymerase chain reaction, Sry, Fmr-1, elk, moose, white-tailed deer, sex identication, wildlife forensic science.

Introduction

Selective harvest programs of game species of Cervidae utilize validation tags for animals of specific age and sex. The hunter is responsible for providing evidence of both age and sex of the killed animal that is consistent with the validation tag. The evidence required for demonstrating the sex of the animal is often the hind leg with attached genitalia or other sex-specific morphological characteristics such as antlers. Hunters that have taken an animal that is inconsistent with their validation tag may claim that the animal is in fact of the legally assigned gender although the sex organs are absent.

In the absence of sex organs, tissue and bone samples from the harvested animal that are associated with the hunter can be used to determine the sex of the animal. Methods of determining the sex of cervids include analysis of testosterone levels [*1*] and the morphology of pelvic girdles [*2*]. The determination of gender of white-tailed deer using testosterone levels proved useful in distinguishing the sex of adult deer but the range of concentrations in male white-tailed deer fawns overlapped with the those observed in does [*1*]. The levels of testosterone were also substantially different among white-tailed deer from different geographic regions. The use of pelvic morphology in white-tailed deer provided a 95% accuracy in sex identification partially as the result of marginal sexual dimorphism among a number of deer pelvises [*2*]. Also this cannot be used to sex fawns in white-tailed deer. We have developed a DNA marker system for the sex identification of tissue samples from three cervid species: elk (*Cervus elaphus canadensis*); moose

(*Alces alces*); and white-tailed deer (*Odocoileus virginianus*). The protocol amplifies sex-specific DNA using the polymerase chain reaction (PCR) and is not dependent on age class and morphological or physiological variation.

The DNA marker we selected to assign gender to an unknown tissue sample originating from elk, moose or white-tailed deer was a region in the sex-determining gene on the Y-chromosome (Sry). A PCR reaction designed to amplify this gene allows sex identification based on the presence of a product in a male and the absence of a product in a female. The Sry gene encodes a protein that has been identified as the testis-determining factor in mammals [*3*]. A phenotypically male deer must have this gene and therefore its identification eliminates any ambiguity with respect to the assignment of gender. Previous wildlife studies have used the Sry gene as a DNA marker for sex identification in a range of terrestrial and marine mammals [*4,5,6*]. To our knowledge this is the first study to utilize the Sry gene in the determination of gender in elk, moose and white-tailed deer for enforcement purposes.

As mentioned above, sex identification techniques that utilize PCR to amplify DNA on the Y-chromosome generate a male-specific product. To confirm that the lack of a product in a reaction is the result of the absence of a Y-chromosome and not the result of a failed reaction a second product present in males and females is simultaneously amplified. This second product acts as an internal control and can be detected using two approaches: 1) the amplification of two homologous loci, one on the Y-chromosome and

the other on the X-chromosome, that demonstrate chromosome-specific differences, e.g. presence or absence of a restriction enzyme site [*4, 7*]; and 2) a multiplex reaction which utilizes two primer sets to amplify both the diagnostic region of interest, e.g. Sry, and another locus as a control to confirm that the amplification conditions were successful [*8*]. We have adopted a multiplex PCR approach for the sex identification elk, moose and white-tailed deer using Sry primers based on ungulate species, i.e. sheep and cattle [*9*] and the human Fragile X gene sequence [*10*] to act as an internal control.

Materials and Methods

Samples

The samples for this study were obtained from several Canadian provincial ministries involved in the management of natural resources. These samples were of known sex as they were collected from check stations during hunting seasons and had intact sex-specific morpholoical characters present. Samples involved in a wildlife forensic case requiring sex identification were submitted from a conservation officer in the Ontario Ministry of Natural Resources. The case involved three samples confiscated from a hunter who possessed a female moose tag and was suspected of harvesting a male moose. A human male and female DNA sample were included in the analyses.

DNA Extraction

Tissue samples were preserved in a lysis buffer (4 M urea, 0.2 M NaCl, 0.5% n-lauroyl sarcosine, 10 mM CTDA (1,2-cyclohexanediamine) 0.1 M Tris-HCl pH 8.0) (Applied Biosystems Inc.). Tissue samples (0.5 g) were ground in liquid nitrogen with 3.5 mL lysis buffer. Samples were incubated at 37°C for up to one week. Proteinase K (62.5 U; Applied Biosystems Inc.) was added to each sample and the samples were incubated at 37°C for an additional 12-24 hours. DNA was purified by two phenol:chloroform (70:30) extractions and one chloroform:isoamyl (24:1) extraction, then precipitated by the addition of 0.1x volume of 10 M ammonium acetate and 2x volume of 95% ethanol. Precipitates were centrifuged at 3000 x g for 15 minutes, washed with 70% ethanol and recentrifuged. The DNA was dissolved in 1x TNE_2 (10 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, 2 mM EDTA (disodium ethylene diamine tetraacetate•2 H_2O). The amount of DNA in a sample was measured using a TK 100 mini-fluorometer using a dye (Hoechst 33258).

PCR Amplification

Approximately 100 ng of DNA was used as template for the amplification of the Sry region [*9*] of the Y chromosome and the 5' region of the Fmr-1 gene [*10*]. The primer sequences used were:

SRY 1 `5' cttcattgtgtggtctcgtg 3'`

SRY 2 5′ cgggtatttgtctcggtgta 3′

FMR-1(c) 5′ gctcagctccgtttcggtttcacttccggt 3′

FMR-1(f) 5′ agccccgcacttccaccaccagctcctcca 3′

Reactions were performed in a total volume of 50 ul under the following conditions: 10 mM Tris-HCl pH 8.4, 50 mM KCl, 0.001% Triton X-100, and 1.5-2.0 mM $MgCl_2$ and 1-2 U of Taq polymerase (Perkin Elmer Cetus). Amplification was performed under a temperature regime of: 94 °C for 5 minutes, 55-60 °C for 30 seconds, and 72 °C for 30 seconds for 1 cycle; 94 °C for 15 seconds, 55-60 °C for 30 seconds and 72 °C for 30 seconds for 30 cycles; and 94 °C for 15 seconds, 55-60 °C for 30 seconds and 72 °C for 5 minutes for 1 cycle. A negative control without DNA was included in each reaction set. Table 1 provides details on the specific conditions for $MgCl_2$ concentrations, Taq polymerase concentrations and annealing temperatures for each of the three cervid species. The reaction conditions for amplifying the Sry product for DNA sequencing are as described above with a 60 °C annealing temperature.

An investigation involving a harvested moose involved DNA analysis to determine the gender of the moose which was taken by a hunter suspected of violating his validation tag. The multiplex protocol for moose was performed on the samples with the addition of a second reaction to confirm the multiplex results. A reaction mix of 100 µl per reaction with Sry primers was added to 200 ng of template DNA. A 50 µl aliquot of this reaction mix plus template was added to a control tube containing 100 ng of male moose

DNA and the remaining 50 µl was amplified with out modification. The temperature regime was as described above using a 60 °C annealing temperature. Both mixed and non-mixed samples were processed simultaneously.

Electrophoresis

PCR products were analysed by gel electrophoresis on agarose gels. The amplified DNA was electrophoresed through 1.0% agarose gels at 80 V for 3-4 hours in Tris-borate buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Flanking molecular weight size standards of 123 b.p. ladder (BRL) were run on each gel. The PCR products were stained with ethidium bromide and visualized under ultra-violet (UV) light. Following successful amplification of a PCR product representing the target region of the Sry product, the fragment was excised from the agarose gel. The amplified product was re-amplified in triplicate to generate enough product for DNA sequencing. PCR products were isolated through a 1.5% low-melting point agarose gel in Tris-acetate (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8). The PCR products were excised from the LMP gel and combined with an equal volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The PCR products were purified using a phenol-chloroform and chloroform extraction and ethanol precipitation and dissolved in 30 µl of sterile ddH_2O.

DNA Sequencing

Two hundred to three hundred nanograms of amplified product was used for cycle sequencing according to the PRISM^{TM} Ready Reaction Dye Deoxy Terminator Protocol (Applied Biosystems Inc.) (MOBIX Facilities, McMaster University, Hamilton, Ontario). The sequencing reaction was performed on a Perkin-Elmer Cetus thermal cycler, Model 480 and the DNA Sequencing System, Model 373A (Applied Biosystems Inc.). The product was amplified with both primers to confirm the sequence using both strands. All the reagents for Cycle Sequencing of the products were combined to a final volume of 20 µl; 9.5 µl terminator premix (fluorescent -tagged dITTP, dATP, dTTP and dGTP, Tris-HCl, pH 9.0, 4.21 mM $(NH_4)_2SO_4$, 42.1 mM $MgCl_2$, 0.42 U Amplitaq DNA polymerase (Perkin-Elmer Cetus), 5.0 µl DNA, 3.2 pmol primer and sterile ddH_2O. DNA sequences were analyzed by the computer program Image Quantification (Molecular Dynamics).

Results

A 139 b.p. Sry fragment and an Fmr-1 product of approximately 350 b.p. were amplified under the appropriate reaction conditions (Table 1) for male elk (*Cervus elaphus canadensis*), moose (*Alces alces*) and white-tailed deer (*Odocoileus virginianus*) and only an Fmr-1 fragment was visible with female samples of the same species. The results were consistent with several elk samples of both sexes (Fig. 1) as well as moose

(Fig. 2) and white-tailed deer (data not shown). The $MgCl_2$ concentrations and annealing temperatures were modified for each cervid species to reduce the non-specific amplification of additional products and to optimize distinct Sry and Fmr-1 products in the multiplex reactions (Table 1). DNA from male and female samples were amplified under the same multiplex reactions used in the amplification of cervid DNA and revealed non-specifc banding patterns with no discrete fragments consistent with human SRY and FMR-1 products.

The DNA sequence of the region of the Sry gene for elk, moose and white-tailed deer demonstrated a high degree of sequence homology to each other, an Asian deer species and to the bovine Sry gene sequence (Fig.3). The sequence homology among the three North American cervids was calculated at 97.8-99.3% and the homology between these cervids and the Asian *Cervus* species was 97.8-98.6%. The sequence homology between the cervid species and the bovine Sry gene sequence was calculated at 93.4 - 95.0%. The sequence homology between the bovine Sry gene and the human SRY gene at this region was observed to be 81.3% [*6*]. The Fmr-1 product was approximately 350 b.p. which is in the size range observed for this region in the human FMR-1 [*10*], the same region in mouse [*11*] and other mammalian species [*12*]. This region is located in the 5' untranslated region of the gene and contains the highly polymorphic CGG trinucleotide repeat structure which have been associated with the Fragile X syndrome [*10*]. No

TABLE 1: Summary of amplification conditions for Sry/Fmr-1 multiplex reactions.

CERVID SPECIES	$MgCl_2$ Concentration	Taq Polymerase (units)	Annealling Temperature
Elk *Cervus elaphus*	2.0 mM	1.0 U	60 °C
Moose *Alces alces*	1.5 mM	1.0 U	55 °C
White-tailed Deer *Odocoileus virginanus*	2.0 mM	1.0 U	60 °C

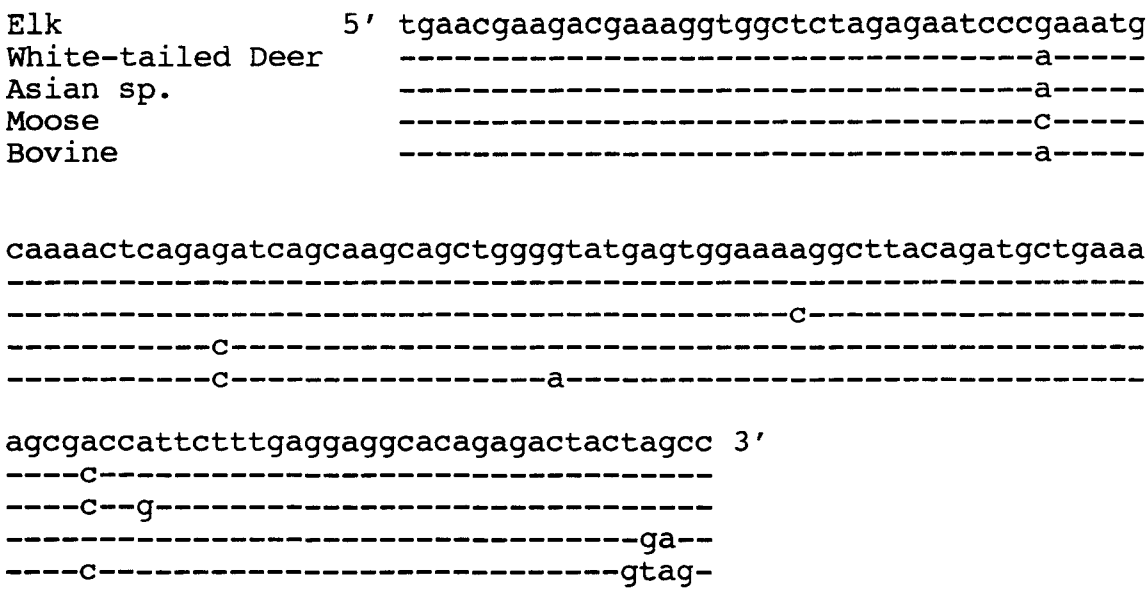
FIGURE 1. Ethidium stained agarose gel (1.2%) of multiplex PCR products from elk (*Cervus elaphus*, Cel) male (m) and female (f) DNA. The Sry and Fmr-1 products are identified. Primer-dimer artefacts can be visualized in the negative control. Female samples demonstrate the Fmr-1 product alone and the male samples demonstrate both the Sry product and the Fmr-1 product. Sizes are shown in base pairs (b.p.) with a 123 b.p. ladder (BRL).

Fmr-1
Sry
Cel (f)
Cel (m)
Cel (m)
Cel (m)
Cel (f)
Cel (f)
Cel (m)
Cel (m)
Cel (m)
Cel (f)
Negative Control
369 b.p.
246 b.p.
123 b.p.

FIGURE 2. Ethidium bromide stained agarose gel (1.2%) of multiplex PCR products from moose (*Alces alces*, Aal) male (m) and female (f) DNA. The Sry and Fmr-1 products are identified. Primer-dimer artefacts can be visualized in the negative control. Female samples demonstrate the Fmr-1 product alone and the male samples demonstrate both the Sry product and the Fmr-1 product. Sizes are shown in base pairs (b.p.) with a 123 b.p. ladder (BRL).

Fmr-1
Sry
Aal (m)
Aal (m)
Aal (m)
Aal (f)
Aal (m)
Aal (m)
Aal (m)
Aal (f)
Aal (m)
Aal (f)
Negative Control
369 b.p.
246 b.p.
123 b.p.

FIGURE 3. DNA sequences of a 139 b.p. region of the Sry gene from elk (*Cervus elaphus canadensis*), white-tailed deer (*Odocoileus virginianus*), an Asian deer species (*Trangulus javanicus*, Genbank Accession Number D13463), moose (*Alces alces*) and Bovine (*Bos taurus domesticus*)[9]. Dashes indicate sequence identity with the elk sequence. Nucleotide differences are outlined according to species.


Elk 5′ tgaacgaagacgaaaggtggctctagagaatcccgaaatg
White-tailed Deer ----------a-----
Asian sp. ----------a-----
Moose ----------c-----
Bovine ----------a-----
caaaactcagagatcagcaagcagctggggtatgagtggaaaaggcttacagatgctgaaa
----------c----------
----------c----------a----------
agcgaccattctttgaggaggcacagagactactagcc 3′
----c----------
----c--g----------
----------ga--
----c----------gtag-

variation in the number of trinucleotide repeats was observed in the amplified products visualized on ethidium bromide stained gels.

The sex identification protocol we developed has been applied to wildlife forensic investigations. One case involved the testing of moose meat associated with an individual suspected of harvesting a bull moose while he possessed a cow moose tag. These details were revealed following the analysis of the case samples. The sex of the moose could not be assessed morphologically due to the lack of sex organs following the processing of the animal and three samples of butchered meat were confiscated from the suspect. The results of the multiplex reaction indicated that the case samples in question were in fact of female origin (data not shown). The results of the multiplex reactions were confirmed by adding known male moose DNA to aliquots of a reaction mix containing only the Sry primers. No Sry product was visible in the original un-modified reactions and an Sry product was visible in the modified samples that contained the male moose DNA (Fig.4) indicating the absence of the Sry gene in the case samples and the absence of any inhibitors which may have prevented the amplification of the male-specific product.

Discussion

The DNA amplification of conserved gene sequences on the Y and X chromosomes through a multiplex PCR reaction allowed an accurate identification of gender from tissue samples of the cervid species investigated (Fig. 1 and 2).

FIGURE 4. Confirmation of the multiplex results in the wildlife investigation utilized the Sry primers in identical reaction mixtures to amplify the template DNA of the case samples in one reaction set (Standard Reactions) and amplify the template DNA with added male moose DNA in the second reaction (Modified Reactions). The ethidium bromide stained agarose gel (1.2%) revealed the presence of the Sry product in the male moose control, Aal (m) and the absence of any product in the female moose control, Aal (f), and case samples 1-3 in the unmodified reaction set (Standard Reactions). The modified reaction set containing male moose DNA all contained an Sry product.

Sry
Aal (m)
Aal (f)
Case Sample 3
Case Sample 2
Case Sample 1
Standard Reactions
Aal (m)
Aal (f)
Case Sample 3
Case Sample 2
Case Sample 1
Modified Reactions
Negative Control
369 b.p.
246 b.p.
123 b.p.

The identification of the gender of a sample originating from a cervid species at the genetic level eliminates the variability which has been observed in other methods of sex determination in deer [*1,2*]. The DNA sequence which was selected to identify the sex of a cervid was the sex-determining region Y gene (SRY) which has been characterized as the testis-determining factor in mammals [*3*] and therefore is essential in determining maleness. The SRY gene encodes a high mobility group protein (HMG) whichdemonstrates a high sequence conservation among mammal species. Designing primers based on the bovine Sry gene, specifically the HMG box region [*9*], allowed the amplification of this gene in other ungulate species. The high degree of sequence homology, i.e. greater than 93%, between cervid and bovine Sry sequences (Fig. 3) confirms the conservation of the HMG box region of this gene which includes the primer sequences.

A region of the FMR-1 gene was selected as the internal control to confirm the female identity of a sample in the absence of an Sry product. Inhibitors to PCR have been identified in samples involved in forensic investigations [*13*], therefore, the amplificaton of only the Sry region could potentially result in the false assignment of a male sample as female given an unsuccessful reaction. The absence of both Sry and Fmr-1 fragments indicates an inconclusive result for that particular multiplex reaction.

Fmr-1 maintains several advantages in its application as an internal control in a multiplex reaction with Sry primers. The first is the high homology of the Fmr-1 gene,

greater than 95% sequence similarity between the human FMR-1 and the mouse Fmr-1 gene [*11*], and this sequence conservation allowed the cross-species amplification of the developed primers. The primers utilized in this study have successfully amplified Fmr-1 in a range of mammalian species [*12*]. The second advantage is the size of the Fmr-1 amplified product in comparison to the Sry product which is potentially important in severly degraded DNA samples. A degraded DNA sample which contains fragments of less than 350 b.p. could amplify the Sry product but not the Fmr-1 product. If the product sizes were reversed and DNA degradation in a male sample prevented the amplification of the Sry product but not the internal control then a false-positive result of female would result. Although the amplification of DNA from highly degraded and ancient sources of DNA [14] indicates this concern is not likely to be an issue in most cases the presence of this safeguard increases the confidence in interpreting and presenting the results. A third advantage is the copy number of Fmr-1 which is equal to Sry in a male sample (1:1) and therefore the competition between the amplification of the two loci is reduced. Mitochondrial DNA (mtDNA) sequences have been applied as an internal control in other multiplex reactions with Sry primers, however, the high copy number of mtDNA sequences compared to nuclear DNA can result in preferential amplification of the internal control [*4*].

DNA from human males and females did not amplify any distinct SRY or

FMR-1 products under the reaction conditions for the three cervid species examined in this study and only non-specific PCR products were visible. The SRY and FMR-1 products could likely be amplified under different conditions, but the optimized reaction conditions established for the cervid species eliminates the issue of false-positives being generated due to DNA contamination from human sources. The specifity observed in the reaction conditions for each cervid species demonstrates the need for the optimization of the protocol on a species-by-species basis.

The application of this protocol to wildlife investigations has been successful. One example proved a sample originated from a female moose, which was consistent with the validation tag of the hunter. An additional step confirmed the multiplex reaction by amplifying the Sry of male moose DNA mixed with the original DNA sample (Fig.4) and confirmed the absence of any inhibitors which may preferentially prevent the amplification of the Sry product. Preferential inhibition of only one product in a multiplex reaction is unlikely as inhibitors that are co-purified with the template DNA prevent the function of Taq polymerase [*13,15*]. This confirmation is not standard in our laboratory when analysing wildlife cases requiring sex identification, but this case was the first investigation utilizing the multiplex sex identification protocol and the additional steps were developed to support the establishment of the protocol in the event the evidence was presented in a court room. The evidence supported the suspect's claim and obviously did not require the presentation of the results in court. Subsequent

investigations have been analyzed using the multilpex protocol to identify samples originating from both male and female cervid samples in violation of the validation tags of the suspect.

The enforcement of the validation tag system is an important herd management practice. Populations of harvested ungulate species can be very sensitive to skewed sex ratios causing a reduction in the number of young born [*16*]. We have developed an accurate and rapid method of determining the gender of harvested game animals which could be incorporated as a standard test for animals processed at check stations during hunting seasons. The standard testing of tagged animals will act as a effective deterant against hunters violating the validation system.

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CHAPTER 5

DEVELOPMENTS OF PROTOCOLS FOR THE ANALYSIS OF DEGRADED AND SMALL QUANTITIES OF DNA IN WILDLIFE FORENSIC INVESTIGATIONS

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Abstract: Wildlife forensic investigations often require that the species of origin be determined for a tissue sample lacking characteristic morphological features. DNA markers have been established to identify the species from tissue samples (Cronin *et. al.* 1991, Bartlett and Davidson 1992, Blackett and Keim 1992, Waye and Haigh 1992, Guglich *et. al.* 1994) in investigations involving illegal commercialization and poaching. These techniques require amounts of high quality DNA not always observed in samples collected in forensic investigations which can yield poor quality or low concentrations of DNA. Approximately 20% of cases submitted for DNA testing have included samples of this type. Examples of these samples include: dried blood stains on clothing or knives; hair; rotting meat; cooked or prepared meat; and samples which contain contaminants such as dyes or organic material. The objective of this project was to establish DNA protocols which will successfully determine the species from forensic samples which yield poor quality or insufficient DNA.

Wildlife investigations in Ontario requiring species identification have predominantly involved white-tailed deer (*Odocoileus virginianus*) and moose (*Alces alces*) with a lesser number of cases involving black bear (*Ursus americanus*). Also the following fish species have been identified by conservation officers and biologists in the Ontario Ministry of Natural Resources as requiring DNA data bases for species identification: yellow perch (*Perca flavescens*); walleye (*Stizostedion virtue*); and brown trout (*Salmo sp.*). Initial DNA data bases have been established for a mitochondrial DNA

(mtDNA) marker, specifically the cytochrome b gene, for the above species using a polymerase chain reaction (PCR) based protocol. The amplification of a specific region of DNA using PCR allows the successful analysis of forensic samples which yield small quantities of degraded DNA. This species identification procedure allows a rapid, reliable and reproducible determination of the species of origin of biological specimens.

INTRODUCTION

Tissue samples which are fresh or well preserved will yield sufficient amounts of high molecular weight (HMW) DNA following extraction protocols. However, the post-mortem decay of animal tissues results in the activity of nucleases, i.e. enzymes that digest DNA, which will reduce the quantity and quality (molecular weight) of DNA (Linn 1981). The amount of DNA degradation is dependent on both the period of post-mortem decay prior to preservation, and the source of the sample, e.g. DNA degradation occurs in the liver, kidneys and stomach in less than one week while the brain cortex can maintain HMW DNA greater than 3 weeks (Bar *et.al.* 1988, Ludes *et. al.* 1993). The rate of DNA degradation can be further increased by high temperatures, high humidity and bacterial contamination (Bar et al. 1988). Forensic samples can yield degraded DNA of less than 1,000 b.p. (Bar *et. al.* 1988, McNally *et. al.* 1989) in size. The polymerase chain reaction (PCR) is a molecular technique which can be applied to the analysis of low yields of poor quality DNA.

PCR replicates specific regions of DNA under laboratory conditions to provide numerous copies of a DNA marker for analysis (Watson et al. 1992). The reaction is initiated by primers which are DNA sequences between 20-30 b.p. in length that are homologous to sequences on either side of the target sequence of interest. The reaction undergoes a cyclic temperature regime resulting in the amplification of the target region of DNA. The reaction undergoes between 25-35 cycles of this temperature regime and each

newly generated DNA target region can be exponentially replicated to generate sufficient material for analysis. Successful amplification of mtDNA sequences has been achieved from historical samples older than 1000 years old (Pääbo *et. al.* 1989), hair and fecal samples (Kohn *et. al.* 1995) and single sperm cells (Lewin et al. 1992).

A PCR based DNA marker for species identification requires a region of DNA which can be amplified from LMW DNA and demonstrates minimal DNA sequence variation within a species, low intra-specific variation, and considerable DNA sequence variation between a species, high inter-specific variation. DNA which is consistent with this criteria and which was selected for this study was a region located on the mitochondrial DNA (mtDNA). MtDNA is a separate molecule localized in the organelle and is distinct from the chromosomes located in the nucleus of cells and can is found at copy numbers of 100-1,000 per cell. MtDNA is in the range of 15,000-17,000 b.p. or 15-17 kilobases (k.b.) in size. MtDNA has mutation rates 5-10 times higher than nuclear DNA since mtDNA polymerase, the enzyme responsible for DNA replication, lacks proofreading activity (Avise *et. al.* 1987). For this reason mutations, i.e. base pair substitutions, will accumulate on the mtDNA between taxonomic units more rapidly than nuclear chromosomes over evolutionary time. The genes localized on the mtDNA demonstrate differences in evolutionary rates (Brown *et. al.* 1985) so a specific region of mtDNA was selected. The gene most appropriate with respect to consistency with the criteria for a species-specific DNA marker was the cytochrome b gene and has been

suggested as a potentially useful marker in species identification in wildlife investigations (Bartlett and Davidson, 1992).

Cytochrome b is a gene which codes a protein involved in the electron-transport chain (Hatefi 1985). The tempo and mode of evolution have been well established for this gene (Kocher *et. al.* 1989, Irwin *et. al.* 1991) as well as the structure and function of the protein (Hatefi 1985). Cytochrome b is an appropriate DNA marker for species identification because the gene is evolutionarily conservative and sequences have been observed to maintain a high level of species specificity with low levels of intra-specific variation and high levels of inter-specific variation (Kocher *et. al.* 1989, Irwin *et. al.* 1991, Hughes *et. al.* 1993, Lento *et. al.* 1994, Zhu *et. al.* 1994). Despite the inter-specific sequence divergence the cytochrome b gene was analyzed in these studies using "universal primers" which have been developed to amplify homologous sequences of this gene (Kocher *et. al.* 1989). Greater than 100 divergent species of mammals, birds, amphibians and fish have been sequenced using the universal primers.

The advantage of universal primers is the development of a standardized method of species identification with a methodology which can be applied across evolutionarily distance species with minimal modifications to the protocol. The primer set that was selected for this study amplifies a 357 b.p. product within the approximately 1,140 b.p. cytochrome b gene. The 357 b.p. fragment includes 50 b.p. of primer sequence from two primers which provides 307 b.p. of gene sequence to be analyzed for base pair differences

between samples. The selection of these primers can accommodate the amplification a region which is in the size range of extremely LMW DNA, i.e. < 1000 b.p. The location of cytochrome b on the mtDNA is also advantageous in analyzing degraded tissue samples due to the high copy number of mtDNA molecules within an individual cell which increases the probability of maintaining an intact 357 b.p. cytochrome b target sequence.

Control samples from fish and wildlife species and the additional species often involved in a wildlife case are required to establish a DNA data base. The DNA data base will store the different cytochrome b sequences for each species and the calculations of sequence divergence between specific species. Data bases will be grouped according to a close association in wildlife forensic investigations: game ungulates and domestic ungulates; black bear and pig; and fish species. The amount of intra-specific and inter-specific sequence variation will determine the level of sequence divergence used to assign the sample to the species of origin and exclude the sample from the remaining species.

MATERIAL AND METHODS

Sample Collection

Samples were provided by Ontario Ministry of Natural Resources conservation officers and biologists. The samples of moose, white-tailed deer and black bear were derived from wildlife forensic investigations, i.e. meat, blood stains and gall bladders, or

harvested animals. The species of the samples involved in forensic investigations were confirmed using established protocols (Guglich *et. al.* 1994) prior to their use as DNA data base samples. Samples of DNA from domestic animals were in storage within a DNA bank at McMaster University. The fish samples were provided by the Lake Erie Management Unit.

DNA Extraction

Samples were preserved in a lysis buffer (4 M urea, 0.2 M NaCl, 0.5% n-lauroyl sarcosine, 10 mM CTDA (1,2-cyclohexanediamine) 0.1 M Tris-HCl pH 8.0) (Applied Biosystems Inc.). Tissue samples (0.5 g) were ground in liquid nitrogen with 3.5 mL lysis buffer. Samples were incubated at 37°C for up to one week. Proteinase K (62.5 U; Applied Biosystems Inc.) was added to each sample and was incubated at 37°C for an additional 12-24 hours. 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 10 M ammonium acetate and 2x volume of 95% ethanol. Precipitates were centrifuged at 3000 x g for 15 minutes, washed with 70% ethanol and recentrifuged. The DNA was dissolved in 1x TNE_2 (10 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, 2 mM EDTA (disodium ethylene diamine tetraacetate•2 H_2O). The amount of DNA in a sample was quantified using a TK 100 mini-fluorometer using a dye (Hoechst 33258).

Polymerase Chain Reaction & DNA Sequencing

DNA served as a template for amplification using the polymerase chain reaction (PCR). The PCR primers used were the universal cytochrome b primers (Kocher *et. al.*, 1989):

Primer 1: 5′ -CCA TCC AAC ATC TCA GCA TGA TGA AA- 3′

Primer 2: 5′ -CCC TCA GAA TGA TAT TTG TCC TCA- 3′

These primers amplify a 305 b.p. region of the cytochrome b gene corresponding to bases 14842-15148 in the complete human mitochodrial DNA sequence. The DNA was amplified under standard PCR conditions in a Perkin-Elmer Cetus Thermal Cycler model 480. The reaction conditions for the enzymatic reaction were 10 mM Tris-HCl (pH 8.4), 50 mM potassium chloride (KCl), 0.001% Triton X-100, 2.0 mM magnesium chloride ($MgCl_2$), 0.2 mM dNTP's, 0.2 μM of each primer and 1.5 U of Taq DNA polymerase (Perkin-Elmer-Cetus) and 100 ng of template DNA carried out in a 50ul volume. Amplification was performed under a temperature regime of: 94°C for 30 seconds, 55° C for 1 minute and 72°C for 30 minute for 30 cycles A negative control consisting of no DNA in the mixture was included as a sample in the reaction set.

PCR products were assessed by gel electrophoresis on agarose gels. The amplified DNA was electrophoresed through 1.0% agarose gels at 80 V for 3-4 hours in Tris-borate buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Flanking molecular weight size standards of 123 b.p. ladder (BRL) were run on each gel. The PCR

products were stained with ethidium bromide and visualized under ultra-violet (UV) light. Following successful amplification of a PCR product representing the target region of the cytochrome b gene, the fragment was excised from the agarose gel. The amplified product was re-amplified in triplicate to generate enough product for DNA sequencing. PCR products were isolated through a 1.5% low-melting point agarose gel in Tris-acetate (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8). The PCR products were excised from the LMP gel and combined with an equal volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The PCR products were purified using a phenol-chloroform and chloroform extraction and ethanol precipitation and dissolved in 30 µl of sterile ddH_2O.

Two hundred to three hundred nanograms of amplified product was used for cycle sequencing according to the PRISM^{TM} Ready Reaction Dye Deoxy Terminator Protocol (Applied Biosystems Inc.) (MOBIX Facilities, McMaster University, Hamilton, Ontario). The sequencing reaction was performed on a Perkin-Elmer Cetus thermal cycler, Model 480 and the DNA Sequencing System, Model 373A (Applied Biosystems Inc.). The product was amplified with both primers to confirm the sequence using both strands. All the reagents for Cycle Sequencing of the products were combined to a final volume of 20 µl; 9.5 µl terminator premix (A-dideoxy, T-dideoxy, G-dideoxy, C-dideoxy with fluorescent dyes tagged, dITTP, dATP, dTTP, dGTP, Tris-HCl, pH 9.0, 4.21 mM $(NH_4)_2SO_4$, 42.1 mM $MgCl_2$, 0.42 U Amplitaq DNA polymerase (Perkin-Elmer Cetus),

5.0 µl DNA, 3.2 pmol cytochrome b primer and sterile ddH_2O. DNA sequences were analyzed by the computer program Image Quantification (Molecular Dynamics).

RESULTS

A 357 b.p. product was amplified using PCR with cytochrome b target primers in moose, white-tailed deer, bovine, swine, black bear, walleye and yellow perch. Successful amplification of cytochrome b sequences was observed in several forensic samples, i.e. tissue from a kill site, blood stains and dried animal parts. The negative controls, i.e. samples without DNA which are processed simultaneously as the data base samples using the same reaction solutions and polymerase, did not produce any amplified products indicating an absence of contamination with other sources of DNA. The sequence comparisons were grouped according to their association in wildlife forensic investigations: game and domestic ungulate species; black bear and pig; and fish species.

The DNA sequences for ungulate species (Fig.1), black bear and pig (Fig.2) and four fish species (Fig.3) were compared to determine the degree of sequence divergence. DNA sequences which have been published in other sources are included in the comparisons when appropriate. The sequence divergence was determined for comparisons between each species and is calculated from the number of base pair differences in the cytochrome b gene sequence. There was considerable divergence

FIGURE 1. DNA sequence of a 307 base pair region of the mitochondrial cytochrome b gene from white-tailed deer, moose, bovine and pig. Dashes indicate sequence identity with white-tailed deer. Nucleotide differences are outlined according to species.

```
White-tailed Deer    5'  ac ttc ggc tct ctg cta gga att tgc tta
Moose                    -- --- --t --- --a t-- --- -g- --- ---
Bovine                   -- --- --- cc- cct -ct --g t-- --- c--
Pig                      -- --- --t --c --c t-- --c --c --- c--


atc cta caa att ctt act ggc cta ttc cta gca ata cat tat aca tcc
--- t-- --- --c --- --a --a --- --- --- --- --- --- --- --- c--
--- --- --- --c --c --a --- --- --- --- --- --- --c --c --- ---
--- t-g --- --c --a --a --- --g --- t-- --- --- --- --c --- --a


gac aca ata aca gca ttt tcc tct gtc act cac atc tgc cga gac gtt
--- --- --- --- --- --c --- --- --- --c --- --- --- --- --t --a
--- --- -c- --- --- --c --- --- --g --c --t --- --- --- --- --g
--- --- -c- --- --t --c --a --a --t --a --- --t --t --- --- --a


aac tat ggc tga att att cga tac ata cat gcc aat gga gca tcc ata
--t --c --- --- t-- --- --- --- --g --- --a --c --- --c --a ---
--- --c --- --- t-c --c --- --- --- --c --a --c --g --t c-a --g
--t --c --a --- g-- --- --c --t c-- --- --a --c --- --- --- ---


ttc ttt atc tgt cta ttc ata cat gtg gga cga gga cta tac tac gga
--- --c --- --c t-- --t --- --- --a --- --- --- --- --- --- ---
--t --- --- --c t-- -at --g --c --a --- --- --c t-- --t a-- --g
--- --- --t --c --- --- --c --c --a --c --- --t --- --- --- ---


tca tat act ttc tta gaa aca tga aac att gga gta att ctc cta ttc
--c --- --- --t c-- --- --- --- --- --c --- --g --c --t --- --t
--t --c --- --t c-- --- --- --- --t --- --g --- g-c --- --g c--
--c --- -ta --- c-- --- --- --- --- --- --- --- g-c --a --- --t


aca gtt ata gcc aca gca ttc gta ggg tac gtc gtc cc   3'
--- --a --- --- --- --- --- -ct --- --t --- --- --
--- --a g-- --- --- --- --t a-- --a --- --- cta --
--c --- --- --a --- --c --- a-- --c --- --- c-g --
```

FIGURE 2. DNA sequence of a 307 base pair region of the mitochondrial cytochrome b gene from black bear and pig. Dashes indicate sequence identity with bear. Nucleotide differences are outlined according to species.

```
Black Bear            5'  ac ttc ggg tcc ctc ctc gga gta tgt tta
Pig                       -- --- --t --- --- t-a --c a-c --c c--

gta cta caa att cta acg ggc cta ttc cta gct ata cac tat aca tca
a-c t-g --- --c --- --a --- --g --- t-- --a --- --t --c --- ---

gac aca act aca gcc ttt tca tca atc acc cat att tgc cga gat gtt
--- --- --a --- --t --c --- --- g-t --a --c --- --t --- --c --a

cac tac gga tga att atc cga tac ata cat gct aac gga gct tcc ata
a-t --- --- --- g-- --t --c --t c-- --- --a --- --- --a --- ---

ttc ttt atc tgc ctg ttc atg cac gta gga cgg ggt ctg tac tat ggc
--- --- --t --- --a --- --c --- --- --c --a --- --a --- --c --a

tca tac cta ctc tca gaa aca tga aac att ggc att atc ctc cta ttt
--c --t a-- t-- ct- --- --- --- --- --- --a g-c c-- --a --- ---

aca gtc ata gcc acc gca ttt ata gga tat gtc cta cc   3'
--c --t --- --a --a --c --c --- --c --c --- g-c --
```

FIGURE 3. DNA sequence of a 307 base pair region of the mitochondrial cytochrome b gene from walleye, yellow perch and largemouth bass (Whitmore *et. al.* 1994) and a 295 base pair region of the cytochrome b gene from brown trout (Giuffra *et. al.* 1994). Dashes indicate sequence identity with walleye. Nucleotide differences are outlined according to species.

```
Walleye                5'   c ctt gga tca cta ctc ggc ctc tgt tta
Yellow Perch                - t-c --- --- --- --t --- --- --- ---
Large Mouth Bass            - --- --c --c --g --g --- --- --c c--
Brown Trout                 - t-- --c --- --c t-a --- --g --- c--


atc acc caa atc ctc aca ggc ctc ttc gtt ata cac tac acc gct gat
--- --- --- --- --a --- --- --- cta -ca --- --- --- --- --a ---
gca --- --- --- --- --- --- t-a ctc -c- --- --- --t --- t-- ---
gc- --- --- --t --t --c --a --- cta -ct --- --- --t --- t-c ---


atc gca aca gcc ttt tca tca gtc gcc cac att tgc cgt gac gta aat
--- --- --- --- --- --- --- --g --- --- --- --- --a --t --- --c
--- -t- --c --a --c --- --c --t --- --- --- --t --c --t --- --c
--- --- --- --- --- --c --t --t tg- --- --- --- --a --t --t -gc


tac ggt tga ctc att cga aat att cat gcc aac ggt gct tct ttc ttc
--- --- --- --t --- --t --- c-- --- --- --- --- --a --c --- ---
--- --- --- --a --- --- --c --- --- --t --t --- --a --c --- --t
--- --- --- --- --c --- --c --- --c --t --- --a --a --- --- ---


ttc ttt atc tgc ata tat atg cat atc ggc cga ggc cta tac tac gga
--t --c --g --- --c --- --- --- --- --- --- --t --g --t --t --c
--- --- --- --- --c --c c-- --- --- --- --g --a t-- --t --t --t
--t --- --- --t --t --- --a --- --- -c- --- --a --c --- --t --t


tcc tac ctc tac tta aaa gag aca tga aat gtg gga gtc atc ctc ctc
--t --t --- --- c-- --g --- --- --- --- a-c --- --a g-- --- ---
--a --- --t --- c-- --- --- --- --- --c a-c --- --a --- --- ---
--- --- --a --t --- --- --a --c --- --- a-c --- --- g-a --g --a


ctt ctc gtt atg act gct gtc gta ggg tat gtc ctg ccc   3'
--- g-a --a --a --c --- tca --t --- --- --- --- ---
--c --a --c --a --- --c t-- --- --- --c --- --- ---
--- --- ac- --a --c --c t-- --g --c
```

TABLE 1: Pairwise percent sequence divergence between ungulate species. Numbers in the top portion of the table are the number of nucleotide differences out of the 307 base pair cytochrome b sequence between species. Percentage differences in base pair composition are presented in the lower portion of the table.

Species	White-tailed Deer	Moose	Bovine	Pig
White-tailed Deer	•	38	52	57
Moose	12.4%	•	63	56
Bovine	16.9%	20.5%	•	76
Pig	18.6%	18.2%	24.8%	•

between game species and domestic ungulates (Table 1); moose and white-tailed deer demonstrated 9.9% and 20.5% sequence divergence, respectively, compared to the bovine cytochrome b sequence and both game species demonstrated approximately 18.0% divergence when compared to pig. A sequence divergence of 12.4% was observed between moose and white-tailed deer and approximately 25% between bovine and swine. The cytochrome b gene sequences were compared for black bear and pig due to their common association in wildlife forensic investigations involving the gall bladder trade. The sequences demonstrated a high degree of variation between the two species with 69 nucleotide differences out of 307 b.p. or 22.5% sequence divergence (Fig.2).

Estimates of intra-specific sequence divergence were calculated for white-tailed deer, moose and bovine by comparing sequences obtained in this study to previously published sequences. The sequence divergence between our white-tailed deer sequences and sequences published by Hughes and Carr (1993), obtained from deer populations in Alberta, was in the range of 0.7-3.2%. The moose sequence we obtained was identical to a previously published sequence (Carr and Hughes 1993). A published sequence of the bovine cytochrome b gene (Anderson 1982) demonstrated 2.1% divergence from our bovine DNA sequence.

Levels of inter-specific sequence divergence were also estimated for several fish species (Table 2). Pairwise percent sequence divergence was found to be lowest among the fish species between walleye and yellow perch at 14.7%. Walleye and yellow perch

TABLE 2: Pairwise percent sequence divergence between fish species. Numbers in the top portion of the table are the number of nucleotide differences in the 307 nucleotides in the cytochrome b gene sequence between species except comparisons with brown trout in which 295 base pairs were compared. Percentage differences in base pair composition are presented in the lower portion of the table.

Species	Walleye	Yellow Perch	Largemouth Bass	Brown Trout
Walleye	•	45	57	66
Yellow Perch	14.7%	•	59	64
Largemouth Bass	18.6%	19.2%	•	66
Brown Trout	22.4%	21.7%	22.4%	•

demonstrated 18.6% and 19.2% sequence divergence, respectively, when compared to largemouth bass. The sequence divergence for a 295 base pair region of the cytochrome b gene of brown trout when compared to the other three fish species were all observed to be greater than 20% different in nucleotide composition. No intra-specific sequence divergence was observed in the comparison of the DNA sequences of the two individual walleye and yellow perch which were sequenced.

DISCUSSION

The amplification of a 357 b.p. product from white-tailed deer, moose, bovine, pig, walleye and yellow perch (Fig. 1,2 and 3) was followed by DNA sequencing of the product. The DNA sequences were confirmed to be the cytochrome b sequence in the six species listed above when compared to published gene sequences of this gene (Kocher *et. al.* 1989, Irwin *et. al.* 1991). Positive identification of the PCR products as cytochrome b confirmed that optimal reaction conditions have been obtained for the above species and that the primer sequences we selected can be applied to a number of species from different taxonomic groups involved in wildlife conservation and enforcement. Therefore an unknown sample collected in a wildlife forensic investigation can be analyzed with a standard protocol to amplify the cytochrome b marker.

Sequence alignments were constructed for those species grouped into DNA data bases according to their association in wildlife investigations. Aligning the cytochrome b gene sequence among ungulate species (Fig. 1) demonstrated substantial levels of sequence

divergence (Table 1) indicating the accumulation of a large number of changes, i.e. base pair substitutions, over evolutionary time. The level of inter-specific variation between moose and white-tailed deer was 12.4% and the game species and domestic cow was 16.9-20.5% which are consistent with the levels observed in a study by Cronin (1991) based on restriction fragment length polymorphisms (RFLP) analysis of total mtDNA. A sequence divergence of 14-20% was observed in a phylogenetic study that examined cytochrome b DNA sequences.

The rate of evolution of cytochrome b has been estimated to be 2.5% per million years (Irwin et al. 1991) and the rate for mtDNA as detected by RFLP analysis is estimated as 2.0% per million years (Wilson *et. al.* 1985). The similarity between pairwise sequence divergence of the ungulate species in this and other studies (Cronin et al. 1991, Irwin *et. al.* 1991) indicates the tempo of evolution for the 307 b.p. region of DNA is consistent with the inter-specific variation previously detected in the mtDNA and specifically the cytochrome b sequence. The 307 b.p. cytochrome b region can therefore be used as an accurate representation of the entire cytochrome b gene and its pattern of evolution.

The cytochrome b sequences of black bear (Shields and Kocher 1991) and pig (Fig. 2) also demonstrated levels of sequence divergence consistent with evolutionary distant mammalian species. These two species are associated in the illegal trade of Ontario black bear gall bladders when suspected poachers or traders claim that galls are pig in origin or

when the swine organ is used as look-a-like bear galls in the trade. The successful amplification of a cytochrome b product from black bear, presently not sequenced, and pig gall bladders indicates the advantage of utilizing a PCR derived DNA marker for investigations involving the trade of animal parts.

The amount of genetic divergence between the two fish species, i.e. yellow perch and walleye, submitted by the Lake Erie Management Unit in the Ontario Ministry of Natural Resources was substantial. The number of nucleotide differences observed in comparing the cytochrome b sequences of these two species and the published sequences of two other fish species (Fig. 3) were in the range of 15-23% (Table 2). The results obtained for the fish species in this study are consistent with other studies in that fish species of different genera have sequence divergences of greater than 17% (Zhu *et. al.* 1994). Zhu *et. al.* (1994) also observed that fish species within the same genera demonstrate divergence of 6-15%.

The amount of sequence divergence at the cytochrome b gene has been observed to be considerably lower within a species than between species at the cytochrome b gene (Kocher *et. al.* 1989). Sequence variation among cytochrome b haplotypes in white-tailed deer has been observed to be less than 4% (Hughes and Carr 1993). One haplotype has been observed in moose across North America in the RFLP analysis of total mitochondrial DNA (mtDNA) demonstrating low levels of genetic variability in the mtDNA (Cronin 1992). Low levels of genetic variability in Ontario moose was also

detected with DNA fingerprinting further demonstrating low levels of intra-specific variation (Guglich *et. al.*, 1993). This was supported by comparing a published moose cytochrome b sequence (Carr and Hughes 1993) with the sequence obtained in this study which demonstrated no variable nucleotides within the 307 b.p. region. Low genetic variability is predicted to be observed in bovine and swine samples due to the highly inbred origins of domesticated species. This is consistent with the low intra-specific divergence observed in the comparison of the bovine cytochrome b sequence obtained in this study to a published bovine cytochrome b sequence (Anderson 1982). Any data bases which contains domestic species should include samples from different breeds and domestic stocks to identify the range of divergence within the domestic species.

Studies of fish populations and sub-species have observed intra-specific sequence divergence of the cytochrome b gene have varied from 4% (Zhu *et. al.* 1994) and have been observed to be as low as 1% (Taylor and Dobson, 1994). A sequence comparison of two walleye cytochrome b sequences revealed a sequence divergence of 2.3%. No sequence divergence was observed in the DNA sequences of two yellow perch which were analyzed in this study. The number of sequences in the data base will be increased to establish more accurate estimates of intra-specific divergence among the fish species.

The sequence divergence which is calculated from the comparison of a forensic sample to control DNA sequences of the species outlined in this study will potentially provide two levels of information depending on the nature of the investigation. The first

is the identification of the species of origin based on low levels of sequence divergence with the appropriate control cytochrome b sequences. The identification of a species from a forensic sample will require a species divergence within the established range observed in the DNA data base. The intra-specific criteria established for each species will accurately identify the species of origin providing control samples for the species exists within the cytochrome b DNA data base. A sequence divergence greater than the range observed in the intra-specific comparisons data base of the control sequence indicates an exclusion from a particular species.

The second level of information provides an exclusion of a sample as originating from a particular species based on a high sequence divergence compared to the control sequence of that species. A suspect will often claim a sample originated from a non-illegal source, e.g. a domestic animal source. Assuming an infraction has occurred, the analysis will provide evidence that the sample is not what the suspect claimed thus contradicting the statement. The exclusion of other species provides evidence by contradicting the suspect's claim. Although advantageous, providing an exclusion from other species is not always possible, e.g. the suspect may not make any claim as to the origin of a sample, and is not essential to the interpretation of the results.

The evolutionary relationship of the species grouped together in a data base are often very distant and the sequence divergence will reflect this distance. Cases may involve closer taxonomic groups where the observed range in the sequence divergence

close to the levels observed for intra-specific comparisons. The ungulate species in this study are at a taxonomic distance which is unambiguous in the sequence divergence. The identification of wild cervid species from game farm species could demonstrate lower inter-specific sequence divergence levels and data bases would have to be established to accurately calculate the narrower ranges in intra and inter-specific variation. Potential overlap in the level of sequence divergence may be a concern in closely related fish species as recent molecular genetic studies have identified misclassification of the previous taxonomic status of certain fish species and sub-species (Seyoum and Kornfield 1992, Taylor and Bentzen 1993).

CONCLUSION

We have examined a method for identifying the species of origin in samples lacking characteristic morphological features which are involved in wildlife forensic investigations. The procedure will provide analysis in samples exposed to environmental insult and post-mortem decay which yield low amounts of poor quality DNA. The protocol offers a standard methodology which can be applied to a wide range of wildlife species. The status of the specific DNA data bases are as follows:

Ungulate Data Base

The ungulate data base demonstrates significant sequence variation between species and low sequence variation within species was observed in comparisons between sequences from previous studies. The observed levels of sequence divergence allows the species identification of samples from cases involving illegal commercialization and poaching of moose and white-tailed deer. PCR products for moose and white-tailed deer samples from a wide range of geographic areas have been obtained and these products will be sequenced to determine the intra-specific variation among several populations. The data base will also be increased in the number of Ontario moose and white-tailed deer sequences to determine the level of variation within the province.

Black Bear Data Base

The high inter-specific sequence variation between black bear and pig will allow a positive exclusion from one or the other species. However, the presence of only one black bear sequence requires that additional bear samples be sequenced to produce an accurate range of the intra-specific sequence divergence. DNA from a black bear gall sample was amplified and the DNA sequence of the cytochrome b product is presently being processed. Additional Canadian black bear samples are presently being obtained by Allan G. Sullivan, Special Investigations Officer for the Ontario Ministry of Natural Resources.

Fish Data Base

The fish species examined in this study also demonstrate high levels of sequence variation among the species. The preliminary analysis of two individual fish of both walleye and yellow perch indicates low intra-specific divergence and increased sample sizes for these two species will be compared to confirm these levels. Species which may be closely related to the species in the data base, e.g. large mouth and small mouth bass, are required to determine accurate levels of sequence divergence. Additional species which may be involved in fishing infractions will also be included in the expanding fish data base.

Upon the establishment of a complete DNA data base for a specific type of wildlife infraction, the levels of intra-specific and inter-specific sequence divergence will allow the assignment of a sample to the species of origin at the exclusion of the other species within the data base. The evidence produced by this protocol will equally support or refute a suspect's claim.

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CHAPTER 6

PINNIPED PENISES IN TRADE: A MOLECULAR GENETIC INVESTIGATION

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Abstract

Molecular genetic techniques were used to identify pinniped penises that were purchased in traditional Chinese medicine shops in Asia (Bangkok, Hong Kong and Shanghai) and North America (Toronto, Calgary, Vancouver and San Francisco). A 261 b.p. region of the cytochrome b gene was sequenced for 21 unknown samples, 3 harp seal control samples and 2 hooded seal control samples. These sequences were compared against published sequences for pinniped species. Fourteen of the purchased samples were identified as pinnipeds: 7 harp seal penises and 4 harp seal testes samples (Shanghai) and 1 other, likely, harp seal penis (Bangkok); 1 hooded seal penis (Vancouver); and 1 Australian fur seal penis (Hong Kong). Seven samples were excluded as originating from pinnipeds, 6 of which were not identifiable to species because of a lack or relevant published control sequences: 2 (1 from Toronto and 1 from Calgary) were most similar to an African canid (*Lyacon pictus*), 3 (2 from Toronto and 1 from Bangkok) were most similar to *Bos taurus* followed by water buffalo (*Bubalis bubalis*), 1(San Francisco) was most similar to *B. bubalis*. One sample (Bangkok) was identified as originating from *B. taurus*. Our results confirm that penises from several different pinnipeds are in international trade. These results confirm that penises from several different pinnipeds are in international trade. The detection of Australian fur seal -- a species which is not legally hunted -- and some unidentifiable species, reveals that trade in legal products is apparently serving as a cover for illegal trade. These findings corroborate

other recent evidence that the lucrative market for pinniped penises may be encouraging the unregulated hunting of seals, including protected species, and the harvesting of unidentified mammalian species.

Introduction

The global trade in wildlife products is a significant one, with estimates of its annual value, both legal and illegal, ranging from US $8 (Geist 1994) to about $20 billion (see Lavigne et al. in press). In many cases, this trade has resulted, and is resulting, in the depletion of numerous species (Ceballos & Brown 1995; Lavigne et al. In Press), particularly mammals. Since 1600, 49% of mammalian extinctions for which causes of extinction have been determined are attributable to human hunting, including for food, skin and feathers, sport, live trade, and the destruction of animals perceived as pests (Groombridge 1992; Lavigne et al. In Press). In the case of marine mammals, specifically, the legacy of overexploitation, primarily in the form of commercial whaling (Gaskin 1982) and sealing (Mowat 1984; Busch 1985), is that many species remain at reduced population levels.

Despite the historical evidence that the promotion of large-scale trade in wildlife products is usually incompatible with responsible wildlife management and conservation (Hewitt 1921; Geist 1988, 1989, 1994; Norse 1993; Robinson 1993; Eltringham 1994; Hoyt 1994; Willers 1994; Lavigne et al. In Press), contemporary attempts at the marketing of marine mammal products are often undertaken in the name of "sustainable utilization" (Baker & Palumbi 1994; Lavigne & Smith 1995). The proponents of this viewpoint argue that if wildlife is to be conserved, it "must pay its own way" (Child & Child 1990, Eltringham 1994) and, therefore, that markets for dead wildlife, including

parts and derivatives, must be promoted on a global scale (IUCN 1992; Rasker et al. 1992; Thomsen 1992; UNCED 1992; Robinson 1993).

Recently, some empirical data was brought to bear on the debate surrounding the "sustainable utilization" of whales that, for the first time, confirmed the extent of the problems associated with trade in these species. In their molecular genetic investigation of the origin of whale meat being marketed in Japan, Baker and Palumbi (1994) produced evidence that the "products available currently on the Japanese retail market may include species that have been imported illegally and others that have been hunted or processed illegally" (p. 1539). They concluded, in an example of the so-called "look-alike" problem in wildlife trade (Lyster 1985; Geist 1988, 1989), that the existence of legal commercial whaling serves as a cover for the sale of illegal products.

Our purpose in the present study was to undertake a similar investigation of international trade in the most valuable product of the "sustainable utilization" of pinnipeds: the penis and attached testes (Bräutigam & Thomsen 1993; RT & Associates 1994; Department of Fisheries & Oceans, Canada 1995b, d). Though the illegal trade in the penises of some species such as Bengal tiger (*Panthera tigris*), primarily for Asian aphrodisiac markets, and the deleterious impact of this trade on wild populations, has been well-documented (e.g. Thapar In Press), the size and value of the trade in seal penises remains unknown. Those species known to be involved include harp seals (*Phoca groenlandica*) hunted by Norway in the Northeast Atlantic (RT & Associates

1994), Cape fur seals (*Arctocephalus pusillus pusillus*) from Namibia, South American fur seals in Uruguay (*A. australis*), and northern fur seals (*Callorhinus ursinus*) in the USA (York 1989, in Bräutigam & Thomsen 1993). Harp and hooded (*Cystophora cristata*) seal penises have also been reported collected by Canadian hunters in the Northwest Atlantic for shipment to Asian markets (RT & Associates 1994; Canadian Press 1995; Department of Fisheries & Oceans, Canada 1995a, b).

We used molecular genetic techniques similar to those employed by Baker and Palumbi (1994) to identify penises purchased in traditional Chinese medicine shops in Asia and North America. This empirical evidence was combined with a systematic review of available knowledge on the international trade in pinniped penises in order to develop an understanding of the scope and value of this commerce and its potential to impact adversely pinniped populations.

Materials and Methods

Selection of DNA Marker

Though Baker and Palumbi (1994) analysed the population-specific variability of the mitochondrial control region to identify the species and geographic origin of the individual whale samples, we selected a region of the cytochrome b gene as the DNA marker to assign unknown samples to species. The reason for this was that our study did not focus on population-specific issues and required a broader survey of mammalian

species potentially involved in the penis trade. Population identification of samples is possible with the control region because of the high mutation rate, which is 3-5x higher than the cytochrome b (Hoelzel et al. 1991), whereas the characteristics of cytochrome b are more consistent with a species-specific marker: low levels of intra-specfic variation and higher levels of inter-specific variation (Kocher et al. 1989, Irwin et al. 1991). Another advantage of cytochrome b was the extensive sequence data base (more than 900 entries in EMBL/Genbank) which already exists for mammals, particularly for pinniped species (Arnason et al. 1995). The combination of the above factors has prompted some authors to propose cytochrome b as a species diagnostic marker in wildlife forensic science (Bartlett and Davidson, 1992).

Sample Collection

Penis samples, in a variety of forms, were purchased at traditional Chinese medicine shops in the following cities: Toronto, Calgary, Vancouver, San Francisco, Bangkok, Hong Kong and Shanghai (Table 1). All of the samples were sold specifically as seal penises.

DNA sequences for harp and hooded seal sequences were obtained from blood (harp 01 and 02; hooded 01 and 02) and muscle (harp 03) from populations in the Northwest Atlantic. Additional control sequences were retrieved from previously published studies and EMBL data bases: Australian fur seal (*A. pusillus*) (Lento et al.

Table 1. Purchased samples indicating geographic origin, type and condition of sample.

Sample	Purchase Location	Sample Appearance
BK01P	Bangkok	Wine containing numerous animal parts and plant material
BK02P	Bangkok	Wine containing cross-sectioned penis and plant material
BK03P	Bangkok	Whole baculum with little tissue
BK04P	Bangkok	Cross-sectional pieces of baculum
BK05P	Bangkok	Cross-sectional pieces of baculum
BK06H	Bangkok	Herbal remedy comprised of various materials
BK07C	Bangkok	Capsules containing red-brown powder
TO01P	Toronto	Whole baculum and testes with little attached tissue
TO02P	Toronto	Whole baculum with no attached tissue
TO03P	Toronto	Thick, long and barbed baculum with attached tissue
CG01P	Calgary	Whole baculum with little attached tissue
SF01P	San Francisco	Whole baculum with little attached tissue
SF02P	San Francisco	Cross-sectional pieces of baculum
SF03P	San Francisco	Whole baculum with little attached tissue
VC01P	Vancouver	Whole baculum with little attached tissue
VC02P	Vancouver	Dried testes

VC03P	Vancouver	Dried testes with part of baculum
VC04P	Vancouver	Whole baculum with attached tissue
VC05P	Vancouver	Cross-sectional pieces of baculum
SH01P	Shanghai	Whole baculum surrounded by tissue.
SH02P	Shanghai	Whole baculum surrounded by tissue
SH03P	Shanghai	Whole baculum surrounded by tissue
SH04P	Shanghai	Whole baculum surrounded by tissue
SH05P	Shanghai	Whole baculum surrounded by tissue
SH06P	Shanghai	Whole baculum surrounded by tissue
SH07P	Shanghai	Whole baculum surrounded by tissue
SH08P	Shanghai	Whole baculum surrounded by tissue
SH09T	Shanghai	Dried testes
SH10T	Shanghai	Dried testes
SH11T	Shanghai	Dried testes
SH12T	Shanghai	Dried testes
HK01P	Hong Kong	Whole baculum with little attached tissue

1995); New Zealand fur seal (*A. forsteri*) (EMBL/Genbank accession number X82293); Antarctic fur seal (*A. gazella*) (X82292); northern fur seal (X82304); ribbon seal (*P. fasciata*) (X82302); ringed seal (*P. hispida*); Weddell seal (*Leptonychotes weddellii*) (X72005); leopard seal (*Hydrurga leptonyx*) (X82297); Hawaiian monk seal (*Monachus schauinslandi*) (X72209); southern elephant seal (*Mirounga leonina*) (X82298); and Hooker's sea lion (*Phocarctos hookeri*) (U12851). Tiger (*Panthera tigris*) (X82301) and bovine (*Bos taurus*) (D34635) sequences were included as controls due to the barbed appearance of one penis and the knowledge that bovine species have been used as substitutes in the trade of animal parts (personal communication, R. Wenting, CITES, Canada).

DNA Extraction

Where possible, $0.2cm^3$ (<0.5g) of tissue scraped off penis bone and testes samples. Blood samples were prepared in 1x lysis buffer (4M urea, 0.2 M NaCl, 0.5% n-lauroyl sarcosine, 10 mM CDTA (1,2-cyclohexanediamine), 0.1 M Tris-HCl pH 8.0). DNA extractions were performed according to Guglich et al. (1994).

Polymerase Chain Reaction

DNA served as a template for amplification using the polymerase chain reaction (PCR). The PCR primers used were the universal cytochrome b primers (Kocher et al. 1989):

Primer 1: 5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3'

Primer 2: 5'-CCC TCA GAA TGA TAT TTG TCC TCA-3'

These primers amplify a 305 base pair region of the cytochrome b gene corresponding to bases 14842-15148 in the complete human mitochondrial DNA sequence. The DNA was amplified in a Perkin-Elmer Cetus Thermal Cycler model 480 under the following reaction conditions: 10 mM Tris-HCl (pH 8.4), 50 mM potassium chloride (KCl), 0.001% Triton X-100, 2.0 mM magnesium chloride ($MgCl_2$), 0.2 mM dNTP's, 0.2 mM of each primer, 1.5 U of Taq DNA polymerase (Perkin-Elmer-Cetus), and 100 ng of template DNA carried out in a 25 ul volume. Amplification was performed under a temperature regime of: 94°C for 30 seconds, 55° C for 1 minute and 72°C for 30 minute for 30 cycles. Samples that gave a low yield of PCR product were increased to 45 cycles to increase the amount of amplified product. The amplified product was re-amplified in triplicate to generate enough product for DNA sequencing. PCR products were isolated through a 1.5% low-melting point agarose gel and excised and purified using a phenol-chloroform and chloroform extraction.

Two hundred to three hundred ng of amplified product was used for cycle sequencing according to the PRISM™ Ready Reaction Dye Deoxy Terminator Protocol (Applied Biosystems Inc.) (MOBIX Facilities, McMaster University, Hamilton, Ontario). The sequencing reaction was performed on a Perkin-Elmer Cetus thermal cycler, Model 480 and the DNA Sequencing System, Model 373A (Applied Biosystems

Inc., Foster City, CA). DNA sequences were analyzed by the computer program Image Quantification (Molecular Dynamics). The product was amplified with both primers to confirm the sequence using both strands.

Analysis of Sequence Variation

DNA sequences from the unknown and control samples, including published sequences for pinniped species, were reduced to 261 b.p. to align the sequences in the computer program Genetic Data Environment (GDE). Sequence variation was estimated using the computer program Phylip 3.5c (Felsenstein 1993). DNA distances of pairwise sequence comparisons were obtained using Kimura's (1980) "two-parameter" model assuming a 2-to-1 transition to transversion substitution in the Phylip program dnadist. Following the calculation of DNA distances for unknown samples compared to the control sequences, the Phylip program neighbor was used to generate a radial Neighbor-Joining tree. The DNA distance and Neighbor-Joining trees, generated for each unknown sample in comparison to the control sequences, established the species or group of species closest in sequence similarity to each penis or testes sample. DNA distances were also calculated for intra-specific pairwise comparisons of the control sequences and additional sequences obtained from EMBL: harp seal (n=3, obtained in the present study); hooded seal (n=4, two samples obtained in the present study, and EMBL accession numbers X82294 and L39209); and New Zealand fur seal (n=3, Lento et al.

1995).

Identification of the unknown samples to the pinniped species of origin was made using the Phylip computer package (Felsenstein 1993). A statistical analysis using the bootstrap method was performed to generate confidence intervals on the phylogenies. The Phylip program seqboot resamples the original data by replacing characters, in this case nucleotides, and estimating the variation among the replicates using the dnadist program. Neighbor-joining trees are calculated for the distance measures of each replicate using the program neighbor and a consensus tree is generated from bootstrap data in the program consense, which provides the confidence interval at each node. The consensus tree is a majority rule tree that is constructed according to the groups or clusters that are generated most often in the replicates. Branch points that were generated in greater than 95% of the replicates were considered as the significant confidence interval (Felsenstein 1985). A total of 500 bootstrap data bases were generated to test the phylogenies. Samples that demonstrated high distance values to the seal control species and did not cluster with the pinniped species in the original Neighbor-Joining tree were assumed to represent samples of non-pinniped origin and were considered separately from the bootstrap analysis.

Kimura's DNA distance (Kimura 1980) was selected as the measure of sequence variation from both approaches in the present study because the algorithm accounts for the increased rate of transitions over transversions that will narrow the range of intra-

specific measurements. The application of this estimate of genetic distance to both approaches also makes the two compatible in supporting the identification of species.

Results

Samples and DNA Quality

DNA was extracted from various portions of the purchased penis and testes samples. A 305 b.p. region of the cytochrome b gene was amplified and sequenced for 21 unknown samples, 3 control samples of harp seal and 2 control samples of hooded seal (aligned in Fig. 1). Sample BK01P was not extracted or amplified since the wine sample contained several species of fish in addition to the cross-sectional penis samples and the probability of contamination was high. The testes samples VC02P and VC03P and samples SH05P and SF02P amplified a small amount of product that did not yield a readable DNA sequence. Samples SF01P, SF03P, VC01P and VC05P were penis bone samples and did not produce an amplified product.

Sequence Analysis

Kimura's DNA distances were calculated for pairwise comparisons of control sequences. The DNA distances for inter-specific comparisons among the seal species were in the range of 0.08-0.20 and greater than 0.20 for comparisons of the seal species to

Figure 1. Alignment of 261 b.p. of the cytochrome b gene obtained from control samples, EMBL sequences and unknown penis and testes samples. Control samples include: harp seal, ribbon seal, hooded seal, ringed seal, Weddel seal, leopard seal, Australian fur seal, New Zealand fur seal, Antarctic fur seal, northern fur seal, sealion, Hawaiian monk seal, southern elephant seal, bovine and tiger.

```
                          1          11         21         31         41        50
                          |          |          |          |          |         |
harp seal 01              tccctgctcg taatctgctt aatcctacag atcctaacag gcctattcct
harp seal 02              .......... .......... ......g... .......... ..........
harp seal 03              .......... .......... .......... .......... ......c...
ribbon seal               .....a..t. g......... .......... ...t...... .t........
hooded seal 01            .....c.... g......... ....t..... .......... ..........
hooded seal 02            .....c.... g......... ....t..... .......... ..........
Australian fur seal        ..t..c.... c.g.a..... .gc.t....a .......... ....t...t.
northern fur seal         ..a..a.... c..ca..... .g..t....a .......... .a........
ringed seal               ..t..t.... g.......c. .........a ...t...... ..t.......
Weddell seal              ..t..t.... g.......c. ....t....a ..t...... ..........
leopard seal              .....c.... cgg.a..... .gc.t....a .......... .t..t...t.
Antarctic fur seal        .....c.... c.g.a..... .gc.t....a .......... ....t...t.
New Zealand fur seal      .....c.... cgg.a..... .gc.t....a .......... ........t.
Hooker's sealion          .....t.... cgg.a..... .gc.t....a .......... ....t.....
Hawaiian monk seal        .....t..a. g.......c. ...t..t..a ...t...... .......t..
southern elephant seal    ..t..c.... g......... g........a .......... ........t.
bovine                    .....c..g. g.......c. .........a .....c.... ..........
tiger                     ........a. g.g.a..t.. .........a ..t..c..c. ....c..t..
SH06P                     ..g....... .......... .......... .......... ..........
SH07P                     .......... .......... .......... .......... ..........
SH03P                     .......... .......... .......... .......... ..........
SH08P                     .......... .......... .......... .......... ..........
SH11T                     .......... .......... .......... .......... ..........
SH12T                     .......... .......... .......... .......... ..........
SH02P                     .......... .......... .......... .......... ..........
SH01P                     .....a.... .......... .......... .......... ..........
SH10T                     .......... .......... .......... .......... ..........
VC04P                     .....c.... g......... ....t..... .......... ..........
SH09T                     ..g....... .......... .......... .......... ..........
SH04P                     ...g..t... .......... .......... .......... ..........
HK01P                     ..t..c.... c.g.a..... .g..t....a .......... ....t.....
BK04P                     .......... g......... ...t...... .......... ..........
CG01P                     ..t.....a. g.g.g...c. g..t...... ..t....... .tt....t..
BK05P                     .....t..a. g.g.a..... ...t.....a ..t..t.... ..........
TO01P                     .....t..a. g.g....... .........a .....c.... ..........
TO02P                     ..t.....a. g.g.g...c. g..t...... ..t....... .tg....t..
TO03P                     .....c..g. g.......c. .......... .....c.... ..........
BK02P                     .a...c..g. g.c.....c. ..........a .....c.... ..........
SF01P                     ---------- ---------- .g....g... .....c.... ..........
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                          51          61          71          81          91          100
                          |           |           |           |           |           |
harp seal 01              ggccatacat  tatacctcag  acacaatcac  agccttctca  tcagtgaccc
harp seal 02              ..........  ..........  ..........  ..........  ..........
harp seal 03              ..........  ..........  ..........  ..........  ..........
ribbon seal               ..........  ..c.......  ......c...  ..........  .....a....
hooded seal 01            a.........  ..........  ......ct..  .....c...g  .....a..a.
hooded seal 02            a.........  .....t....  ......ct..  .........g  .....a..a.
Australian fur seal       a........c  ..c.......  ....c.c...  ......t...  .....c....
northern fur seal         a.....g..c  ..........  ....c.c...  ..........  .....cg...
ringed seal               a........c  ..c.......  ......c...  ..........  .....a....
Weddell seal              a.........  ..c.......  ......ct..  ......t...  .....t..a.
leopard seal              a........c  ..c.......  ....c.c...  ......t...  .....c....
Antarctic fur seal        a........c  ........g.  ....c.c...  ......t...  .....t....
New Zealand fur seal      a........c  ..........  ....c.c...  ......t...  .....c....
Hooker's sealion          a........c  ..........  ....c.c...  ......t...  ..g..c....
Hawaiian monk seal        a........c  ..c.......  ......c...  ......t...  ...a.c..a.
southern elephant seal    a..t......  ..c...c...  ......c...  ......t...  .....c..a.
bovine                    a..a.....c  ..c..a..c.  ......ca..  ...a.....c  ..t..t....
tiger                     a.........  ..c..a....  .......a..  c..t......  .....c....
SH06P                     ..........  ..........  ..........  ..........  ..........
SH07P                     ..........  ..........  ..........  ..........  ..........
SH03P                     ..........  ..........  ..........  ..........  .....a....
SH08P                     ..........  ..........  ..........  ..........  ..........
SH11T                     ..........  ..........  ..........  ..........  ..........
SH12T                     ..........  ..........  ..........  ..........  ..........
SH02P                     ..........  ..........  ..........  ..........  ..........
SH01P                     ..........  ..........  ..........  ..........  ..........
SH10T                     a.........  ..........  ..........  ..........  ..........
VC04P                     a.........  .....t....  ......ct..  .........g  .....a..a.
SH09T                     ..........  ..........  ..........  ..........  ..........
SH04P                     ..........  ..........  ..........  ..........  ..........
HK01P                     a........c  ..c.......  ..g.c.c.g.  ......t...  .....c....
BK04P                     .........c  ..........  ..........  .........c  ..........
CG01P                     a.....g..c  ..c..a....  .t...tca..  ......g...  .....c....
BK05P                     a..a.....c  ..c..a..c.  ......ca..  ...a.....c  ..t..c..t.
TO01P                     a..a.....c  ..c..a..c.  ......ca..  ...a.....c  ..........
TO02P                     a.....g..c  ..c..a....  .t...tca..  .....gt...  .....c.g..
TO03P                     .........c  .....a....  ......ca..  ...a......  ..t.......
BK02P                     a..a.....c  ..c..a..c.  ......ca..  ...a.....c  ..t..t....
SF01P                     a..a.....c  .....a..c.  ......ca..  ...a.....c  ..t..cga..
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                          101        111        121        131        141       150
                          |          |          |          |          |         |
harp seal 01              atatctgtcg agacgtaaac tacggttgaa tcatccgata tctacacgca
harp seal 02              .......... .......... .....c.... .......... c.........
harp seal 03              .......... ...g...... .......... .......... ..........
ribbon seal               .......c.. .......... .....c...t .......... .t........
hooded seal 01            .c........ .......... .........t .t........ ..........
hooded seal 02            .c........ .......... .....c.... .t........ ..........
Australian fur seal       .c..t..c.. ......g... .....c.... .......... .a.g..t...
northern fur seal         ....t..c.. .....ag... .....c.... .......... .a.......c
ringed seal               .c.....c.. .......... .....c.... .t.....t.. ...t......
Weddell seal              .c.....c.. g......... .....c.... ....t..... ca........
leopard seal              .c..t..c.. ......t... .....c.... ....t..g.. ca.g......
Antarctic fur seal        ....t..c.. .......... .....c...g .t..t..g.. ca.g......
New Zealand fur seal      .c..t..c.. ......t... .....c.... .t..t..g.. ca.g......
Hooker's sealion          .c..t..c.. ......g... .....c.... .......... ca.g......
Hawaiian monk seal        .c.....c.. .........t .....c.... .t........ .a.......c
southern elephant seal    .c.....c.. ...t...... ..t..... .......... ca........
bovine                    .......c.. ......g... .....c.... .......... ca........
tiger                     ....t..c.. c......... ..t..c.... .t........ ......t..c
SH06P                     .......... .......... .....c.... .......... ..........
SH07P                     .......... .......... .....c.... .......... ..........
SH03P                     .......... .......... .....c.... .......... ..........
SH08P                     .......... .......... .....c.... .......... ..........
SH11T                     .......... .......... .....c.... .......... ..........
SH12T                     .......... ...t...... .....c.... .......... ..........
SH02P                     .......... .......... .....c.... .......... ..........
SH01P                     .......... .......... .....c.... .......... ..........
SH10T                     .......... .......... .....c.... .......... ..........
VC04P                     .c........ .......... .....c.... .t........ ..........
SH09T                     .......... .......... .....c...g .......... ..........
SH04P                     .......... .......... .....c.... .......... .........g
HK01P                     .c..t..c.. ......g... .....c.... .......... .a.g..t...
BK04P                     .......... .......... ..t..c...g .......... a.....t...
CG01P                     .........a .....c.gt .....c.... .t....at.. .a....t...
BK05P                     .......c.. ...t..c... ..t.ac.... ....tt.... ca........
TO01P                     .........a ...t..c.gt .....c.... .t.....t.. .a....t...
TO02P                     .........a ...t..c.gt .....c.... .t.....t.. .a....t...
TO03P                     .......c.. ......g... .....c...g .......... .a........
BK02P                     .......c.. ......g... .....c.... .......... ca........
SF01P                     .c.ca.c... ......g... ..t..c.... .t........ ca........
```

```
                          151         161         171         181         191         200
                          |           |           |           |           |           |
harp seal 01              aatggggcct  ccatattttc  catctgctta  tacatacacg  tgggacgagg
harp seal 02              .....a....  .........t  ..........  ..........  .a........
harp seal 03              ..........  ..........  ..........  ..........  ..........
ribbon seal               ..c..a....  .........t  ..........  ..t..g..t.  .a........
hooded seal 01            ..........  ..........  .......c.g  ..........  ..........
hooded seal 02            .....a....  .........t  .......c.g  ..........  ..........
Australian fur seal       .....a....  .......c.t  ......tc.c  ..........  .a........
northern fur seal         .....a..t.  .......c.t  t......c.t  ..........  .a........
ringed seal               .....a..t.  .......c.t  .......c..  .....g..t.  .a........
Weddell seal              .....a..a.  .......c.t  .......c..  ..........  .a........
leopard seal              .....a....  .......c.t  ......tc.c  ..........  .a........
Antarctic fur seal        .....a....  .......c.t  ...t..tc..  ..........  .a........
New Zealand fur seal      .....a....  .......c.t  ......tc.c  ..........  .a........
Hooker's sealion          .....a....  .......c.t  ......tc.c  ..t.......  ..........
Hawaiian monk seal        ..c..a..a.  .t.....c.t  .t........  ..........  .a........
southern elephant seal    .....a....  .......c.t  .......c..  .........a  .a........
bovine                    ..c..a..t.  .a..g....t  t.........  ..t..g....  .a........
tiger                     ..c..a....  .......c.t  t......c..  .....g....  .a........
SH06P                     .....a....  .........t  ..........  ..........  .a........
SH07P                     .....a....  .........t  ..........  ..........  .a........
SH03P                     .....a....  .........t  ..........  ..........  .a........
SH08P                     .....a....  .........t  ..........  ..........  .a........
SH11T                     .....a....  .........t  ..........  ..........  .a........
SH12T                     .....a....  .........t  ..........  ..........  .a........
SH02P                     .....a....  .........t  ..........  ..........  .a........
SH01P                     .....a....  .........t  ..........  ..........  .a........
SH10T                     .....a....  .........t  ..........  ..........  .a........
VC04P                     .....a....  .........t  .......c.g  ..........  ..........
SH09T                     .....a....  .........t  ..........  ..........  .a........
SH04P                     .....a....  .........t  ..........  ..........  .a........
HK01P                     .....a....  .......c.t  ......tc.c  ..........  .a........
BK04P                     .....a....  .......c.t  ..........  ..........  .a........
CG01P                     ..ca.a..t.  .......c.t  t......c..  .tt.....t.  .a...t..a.
BK05P                     .....a..t.  .a.....c.t  ..........  ..t.......  .a........
TO01P                     .....a....  .a.......t  ..........  ..t.......  .a........
TO02P                     ..ca.a..t.  .......c.t  t......c..  .tt.....t.  .a...t..a.
TO03P                     .....a....  ....g....t  ..........  ..t..g....  .a........
BK02P                     ..c..a..t.  .a..g.c..t  t.........  ..t..g....  .a........
SF01P                     .....a....  .........t  ...g...c..  ..t.......  .a........
```

```
                        201         211         221         231         241         250
                        |           |           |           |           |           |
harp seal 01            actctactac ggttcctaca cattcacaga aacatgaaat ttcggcatta
harp seal 02            .......... .......... ...c...... .......... a.........
harp seal 03            .......... .......... ...c...... .......... ..........
ribbon seal             ...a...... ..c.....t. .......... .........c a.........
hooded seal 01          ...g...... .......c.. ....t..... g......... ..........
hooded seal 02          ...g...... ..c....... ....t..... g......... a.........
Australian fur seal     ...a.....t ..a..a.... ..c.g..... .......... a.t..t....
northern fur seal       ...g.....t ..a....... ..c....... g......... a.t.....c.
ringed seal             ...g...... ..c....... .......... ........ca a.........
Weddell seal            ...a...... ..c....... .......... .........c a.......c.
leopard seal            ...a.....t ..g....... .gc.a.t... .........c a.t.....c.
Antarctic fur seal      ...a.....t ..a..t.... ..c.a..... .........c a.t.....c.
New Zealand fur seal    ...a...... ..a....... .gc.t.t... .........c a.t.....c.
Hooker's sealion        .t.a...... ..c....... ..c.a..g.. .........c a.t.....c.
Hawaiian monk seal      ...a.....t ..c.....t. .......... .........c a.........
southern elephant seal  ...a...... ..c....... .......... .........c a.........
bovine                  ct.a..t..t ..g..t.... .t..tct... .......... a....ag.a.
tiger                   .a.a...... ..c....... .c...t.... ......g... a.t..a..cg
SH06P                   .......... .......... .......... .......... a.........
SH07P                   .......... .......... .......... .......... a.........
SH03P                   .......... .......... .......... .......... a.........
SH08P                   .......... .......... .......... .......... a.........
SH11T                   .......... .......... .......... .......... a.........
SH12T                   .......... .......... .......... .......... a.........
SH02P                   .......... .......... .......... .......... a.........
SH01P                   .......... .......... .......... .......... a.........
SH10T                   .......... .......... .......... .......... a.........
VC04P                   ...g...... ..c....... ....t..... g......... a.........
SH09T                   .......... .......... .......... .......... a.........
SH04P                   .......... .......... .......... .......... a.........
HK01P                   ...a.....t ..a..a.... ..c.g..... c......... a.t..t....
BK04P                   c......... .......... .......... .......... a.........
CG01P                   ga.a...... ..c......g t.....t... .......g.. a.t..a....
BK05P                   c..a..t..t ..a..a..t. .g..tct... .........c a.t..ag.a.
TO01P                   c..a.....t ..a..a.... .g..tct... .......... a.t..ag.a.
TO02P                   gt.a...... ..c......g t.....t... .......... a.t..a....
TO03P                   ct.a..t... .....t.... .....ct... .......... a....ag...
BK02P                   ct.a..t... ..g..t.... .t..act... .......... a.t..ag.g.
SF01P                   ca.a.....g ..a..a..t. .c..tct... g........c a....ag.a.
```

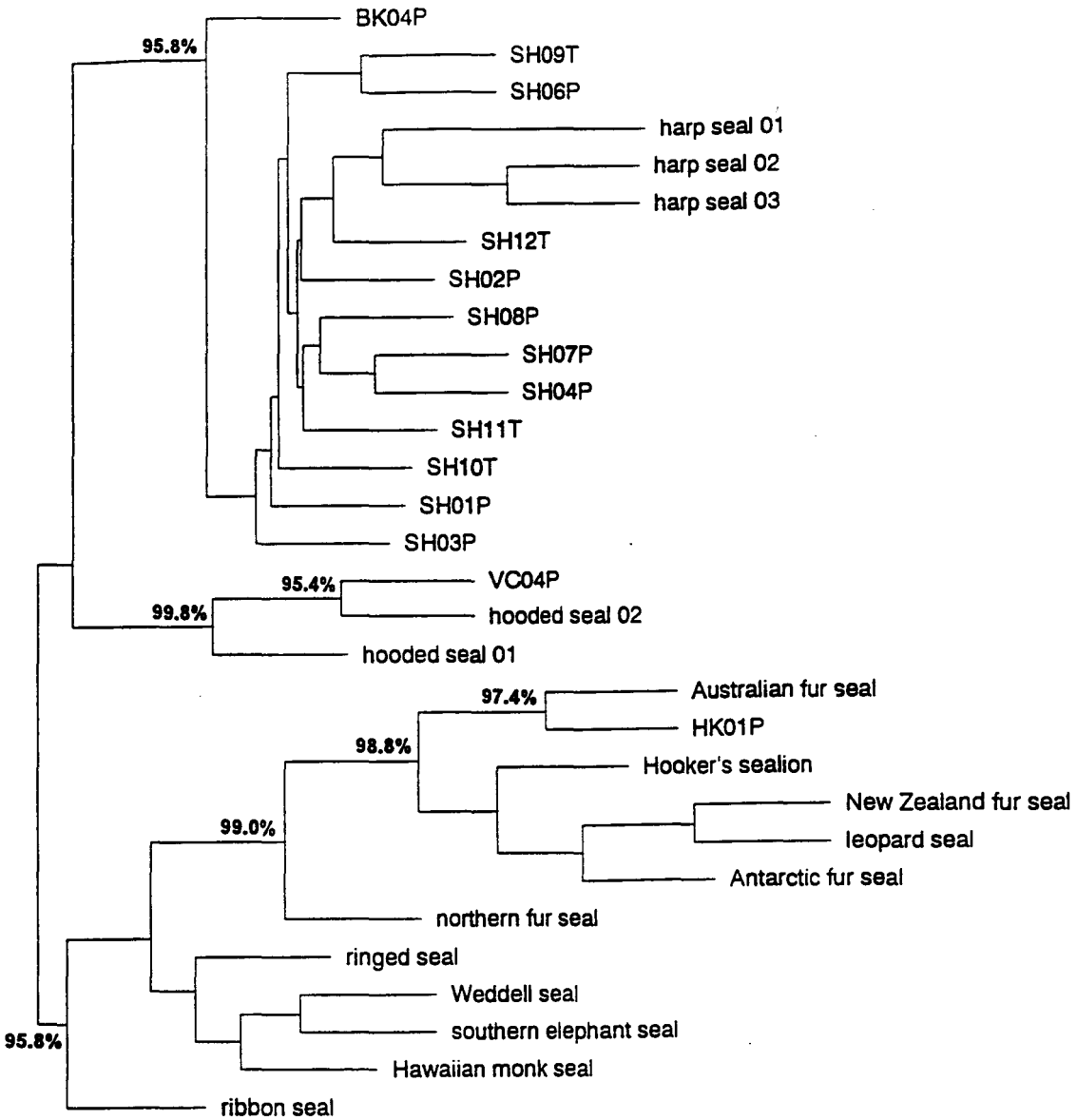
```
                          251        261
                          |          |
harp seal 01              tcctcctatt c
harp seal 02              .......... .
harp seal 03              .........c .
ribbon seal               .t...t.g.. .
hooded seal 01            .....t.... t
hooded seal 02            .....t.... t
Australian fur seal       .......g.. t
northern fur seal         .....t..c. t
ringed seal               .....t.... .
Weddell seal              .t........ t
leopard seal              .......... t
Antarctic fur seal        .......... t
New Zealand fur seal      .......... t
Hooker's sealion          .......g.. t
Hawaiian monk seal        .t...t..c. .
southern elephant seal    .......... .
bovine                    ....t..gc. .
tiger                     .a..at.... t
SH06P                     .......... .
SH07P                     .......... .
SH03P                     .......... .
SH08P                     .......... .
SH11T                     .......... .
SH12T                     .......... .
SH02P                     .......... .
SH01P                     .......... .
SH10T                     .......... .
VC04P                     .....t.... t
SH09T                     .......... .
SH04P                     .......... .
HK01P                     .......g.. .
BK04P                     .......... .
CG01P                     .t...t.c.. t
BK05P                     .......... .
TO01P                     .......... .
TO02P                     .t...t.c.. t
TO03P                     .......g.. .
BK02P                     ....t..gc. .
SF01P                     .......... .
```

the evolutionarily more distant species of tiger and *B. taurus*. Intra-specific DNA distances calculated for harp seal, hooded seal and New Zealand fur seal were in the range of 0.00-0.04.

Fourteen samples grouped closest to one of the seal species and represent penis or testes of pinniped origin. Eleven Shanghai samples had DNA distance values in the range of 0.02-0.05 and one sample, BK04P (Bangkok), demonstrated a value of 0.05-0.08 when compared to the harp seal controls. Sample VC04P (Vancouver) had an identical sequence to hooded seal 02 and a DNA distance of 0.04 when compared to hooded seal 01. Sample HK01P (Hong Kong) demonstrated a DNA distance of 0.02 from the Australian fur seal sequence. The pairwise DNA distances observed between the unknown samples and the remaining seal species were in the range of 0.08-0.20. The radial Neighbor-joining trees derived from Kimura's DNA distances (data not shown) clustered the unknown samples with the control samples demonstrating the lowest amount of sequence variation.

The fourteen samples which demonstrated low pair-wise DNA distance measures with one of the seal species , i.e. in the range of intra-specific comparisons estimated for the seal samples, were assessed with a bootstrap analysis of 500 replicates to generate a consensus tree (Fig. 2). The phylogenetic relationships of the pinniped species observed in the neighbor-joining tree is consistent with the consensus tree. Twelve of the unknown samples grouped with the harp seal controls in a significant

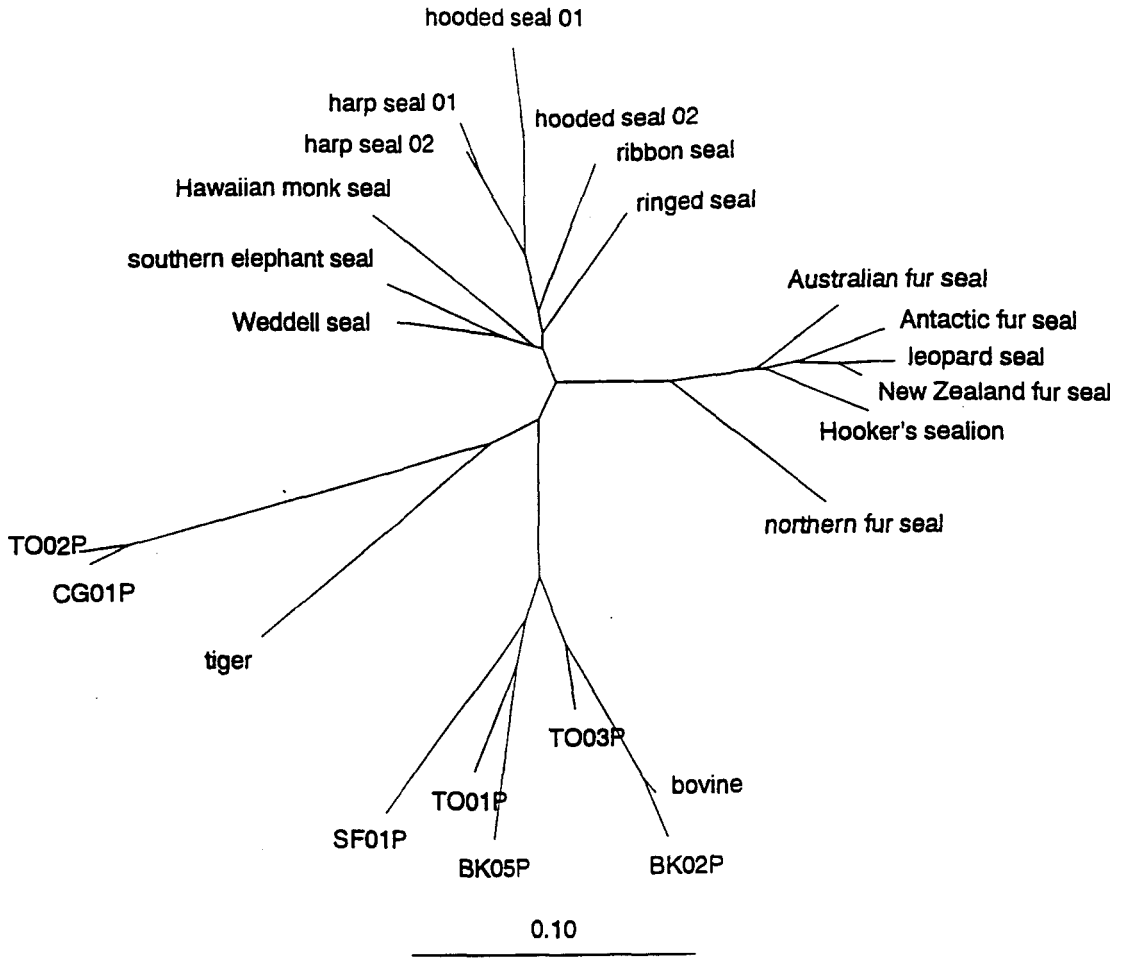
Figure 2. Consensus tree of 500 bootstrap neighbor-joining distance trees. The tree was constructed from the pairwise distance estimates calculated with the computer programs dnadist, seqboot, neighbor and consense contained in the Phylip 3.5c (Felsenstein 1993) package. The number located at the nodes indicates the percentage of bootstrap trees that contain this pattern.


BK04P
95.8%
SH09T
SH06P
harp seal 01
harp seal 02
harp seal 03
SH12T
SH02P
SH08P
SH07P
SH04P
SH11T
SH10T
SH01P
SH03P
95.4%
VC04P
99.8%
hooded seal 02
hooded seal 01
97.4%
Australian fur seal
HK01P
98.8%
Hooker's sealion
New Zealand fur seal
99.0%
leopard seal
Antarctic fur seal
northern fur seal
ringed seal
Weddell seal
southern elephant seal
95.8%
Hawaiian monk seal
ribbon seal

number of trees (95.8%). Although sample BK4P branched away from the main groupings of established harp seal sequences and demonstrated slightly elevated pairwise DNA distance measures with the control harp sequences, the sample grouped within thesignificant confidence intervals of the harp seal lineage. Despite the elevated DNA distance there is a high probability BK04P originated from a harp seal. The sequence did have a higher number of ambiguous nucleotides which may have been interpreted incorrectly (data not shown) or, alternatively, the fact that the sample was cross-sectioned, and may have been packaged with other penis samples possibly resulted in a low level of contamination. Sample VC04P grouped with the hooded seal controls in 99.8% of the replicates. Sample HK01P grouped with the Australian fur seal control sequence in 97.4% of the generated trees in the bootstrap analysis.

Seven samples were assessed at considerable DNA distances from the two clusters of seal control sequences (Fig. 3). Two samples, CG01P (Calgary) and TO02P (Toronto), demonstrated DNA distances of 0.24-0.33, from all the control species. Four samples, BK02P, BK05P (both from Bangkok), TO01P and TO03P (both from Toronto), were observed at a DNA distance closer to the *B. taurus* control sequence (Fig. 3). Samples CG01P and TO02P originated from species not present in the DNA sequence data base. The DNA distance of BK02P to *B. taurus* is in a range consistent with the intra-specific DNA distances observed for seal species at less than 0.04 and a bootstrap analysis produced a significant clustering of the unknown with *B. taurus*

Figure 3. Radial neighbor-joining tree. The tree was constructed from the pairwise distance estimates calculated with the computer programs dnadist and neighbor contained in the Phylip 3.5c (Felsenstein 1993) package. DNA distance is represented by the distance measure provided in the scale.


hooded seal 01
harp seal 01
hooded seal 02
harp seal 02
ribbon seal
Hawaiian monk seal
ringed seal
southern elephant seal
Australian fur seal
Weddell seal
Antactic fur seal
leopard seal
New Zealand fur seal
Hooker's sealion
northern fur seal
TO02P
CG01P
tiger
TO03P
bovine
TO01P
SF01P
BK05P
BK02P
0.10

(96.0%). The observed pairwise DNA distance values for samples BK05P, TO01P and TO03P indicate that the samples are not *B. taurus*, but the range in values is consistent with related species. Sample SF01P produced a smaller sequence at the 5' end and the control sequences were modified in length to provide an accurate measurement of DNA distance. The sample grouped with the *B. taurus* control in a Neighbor-Joining tree (data not shown) at a DNA distance of 0.16 from the *B. taurus* sequence. The sample could not be identified as to species but may be similar in origin to samples BK05P, TO01P and TO03P.

The DNA sequences of samples CG01P, TO01P, TO02P, TO03P, BK05P and SF01P were searched with the BLAST program to identify the cytochrome b sequences of the highest sequence similarity. Samples CG01P and TO02P were most similar to the cytochrome b sequence of an African canine species (*Lycaon pictus*) and the DNAdistances were calculated as 0.18 and 0.21 respectively. Samples TO01P, TO03P and BK05P demonstrated the closest sequence similarity to *B. taurus* followed by the water buffalo (*Bubalis bubalis*) sequence. Sample SF01P demonstrated the closest similarity to the water buffalo sequence, at a DNA distance of 0.10, followed by the *B. taurus* sequence.

Discussion

Identification of Species in Trade

This is the first investigation to confirm the widespread sale of parts from several pinniped species. Our study has also confirmed that the parts of other, as yet unidentified, species, are being sold as pinniped parts, making evident the difficulties associated with identifying and monitoring seal parts in trade. As a result, regulated legal trade serves may serve to create a number of problems, including: providing a cover for unregulated illegal trade; providing opportunities for the substitution of different species (legal or illegal) into trade, and for the substitution of illegal for legal parts. One of the species that was identified, the Australian fur seal, is not legally hunted, which would indicate that the penis was likely derived from illegal activity.

Only one other published study of which we are aware has attempted to identify pinniped penises in trade, and involved the morphological examination of the bacula of purchased penises seized by the US Fish & Wildlife Service in various US and Canadian Pacific ports (Espinoza et al. 1994; B. Yates, National Fish and Wildlife Forensics Laboratory, Ashland, Oregon, pers. comm.). Out of 13 items examined, 10 were found to be from pinnipeds, and the remainder were derived from canids (Espinoza et al. 1994). The US Fish & Wildlife Service has continued to conduct serological tests on seized penises labeled as being of pinniped origin, and has identified specimens of northern fur seal, canids and ungulates (B. Yates, pers. comm.).

There are several possible reasons for our inability to successfully amplify DNA from all of the tissues that were sampled. Environmental conditions such as temperature and humidity will increase DNA degradation in post-mortem tissues (Ludes et al. 1993) as will bacterial contamination by increasing nuclease activity (Bar et al. 1988). The lack of amplified product may have been the result of an absence of tissue or the result of inhibitors in the mixture that prevented amplification; for example inhibitors to PCR have been identified in human forensic samples (Akane et al. 1994). Finally, some samples may have been in contact with additional samples before purchase resulting in contamination of the dried tissues and sequencing error due to the presence of multiple sequences.

The relationship of the seal control sequences generated in the Neighbor-joining and consensus trees demonstrated differences to a previously published phylogeny of pinnipeds using the cytochrome b gene (Arnason et al. 1995) likely because only a portion of the gene was used in the present study. A smaller region of the cytochrome b gene was selected to increase the probability of amplifying a region of cytochrome b from samples possibly containing degraded DNA and, as a result, the variation in the calculation of DNA distance increased as the size of the sequence decreased (Martin et al. 1990). Despite the minor differences in the arrangement of the phylogenetic relationships, the bootstrap values were significant for the deeper branches that separated sequences at the species level in both studies.

The preliminary identification of certain samples as carnivores or ungulates was based on the inter-generic values estimated in the seal data base. These samples may not represent either of these groups and their identity remains inconclusive. This lack of a positive assignment identifies the need for the establishment or additional data bases to include the range of species involved in the animal-parts trade.

There are two types of data bases which can be established which are dependent on the available cytochrome b sequences: the first type of data base includes sequences from the species of interest and their closely related species; and the second type of data base includes sequences from a large number of animals of the species of interest. The two types of data bases correspond to two approaches of species identification: the determination of the phylogenetic relationships of unknown sequences to the control sequences; and a determination of intra-specific DNA distance values. The phylogenetic approach requires a complete set of sequences from related species to allow a positive assignment in an evolutionary context. In the absence of a complete set of species a sample may be falsely identified as a closely related species due to that sequence representing the closest association in the context of the data base. The criteria for species identification using intra-specific DNA distance measures requires sequences from a large number of animals to determine the majority of haplotypes and the appropriate levels of sequence variation within the species. A complete data base of related species is not required for the intra-specific DNA distance criteria as the identification of a species

is dependent on the established ranges of within species variation and not the closest relationship to a sequence within a data base. Ideally both approaches should be used to support the identification. This will be limited, however, by the available sequences in a data base.

The data base we have established for pinnipeds focused on the phylogenetic approach because a relatively complete set of cytochrome b sequences exist for the seal species (Arnason et al. 1995). Comparisons of intra-specific DNA distance estimates for three seal species and inter-specific comparisons of the control samples were also analysed to provide preliminary ranges of sequence variation for different taxonomic levels. These values were used to confirm the phylogenetic placement of the unknown samples in relation to the control seal sequences.

Conservation Implications

Though the available data on the size of the international trade in penises, bacula and testes, including those from pinnipeds, are incomplete, they indicate that the trade is a lucrative one, and is apparently growing in size (Bräutigam & Thomsen 1993, Guardian News Service 1995). For example, Australia annually exports on the order of 5,000 tons of bull penises to the Chinese aphrodisiac market for "$1.10 a kilo plus freight" (Guardian News Service 1995). A single northern fur seal baculum (*C. ursinus*) sold for US $35.00 in 1989, and was reported in 1993 to be worth more than the pelt; one kg of *C. ursinus* bacula comprises over 40 subadult males (A. York, cited in Bräutigam 1989). In 1987,

3,000 units of *C. ursinus* bacula and testes were detained when consigned to a Hong Kong company; in 1988 over 10,000 units were detained (Anonymous 1989). Though there are no reports of international trade in walrus (*Odobenus rosmarus*) penises or bacula (Bräutigam & Thomsen 1993), it is known that they have been sold to tourists in Alaska at US $75.00 a piece (Riewe & Amsden 1980, in Anonymous 1987). Bräutigam and Thomsen (1993) reported that large shipments of Cape fur seal (*A. p. pusillus*) derivatives, reported as illegal, were intercepted on import from Hong Kong into the United States in both 1984 (7,261) and 1988 (10,655). South Africa's export of 45 kg of "bones" to Hong Kong in 1980 was followed by reported exports to the same destination of 530 kg of "specimens" (1986), 133 kg of "bones" (1987), and 778 kg of "bones" (1988). In addition, 112 kg of "specimens" were transported to Taiwan in 1986 (Bräutigam & Thomsen 1993).

Canada has been attempting to expand its hunt for harp and hooded seals in recent years (RT & Associates 1994; Tobin 1994,1995), and in 1994 the total number of harp seal penises reported landed was 4,547 at a unit price per landed penis of CAN $19.91 (Department of Fisheries & Oceans, Canada 1995b). A recently released report that was completed for the government of Canada's Northwest Territories, concluded that there is a large market for seal penises in Asia, particularly China, and quoted one Canadian company that had received offers of US $130.00 per pound for air dried penis with testes attached (RT & Associates 1994). RT & Associates also reported that

Norway supplies almost 50 percent of Hong Kong's current market demand, shipping approximately 8,000 harp seal penises in 1993. The average price paid to sealers for a seal penis over 25 cm long was CAN $26.00, and for 18 to 25 cm long was CAN $20.00. The report also concluded that "there may also be a sizable market for seal organs within Canada, primarily in ethnic communities of the larger cities" (p. 11), and that out of all the possible seal products examined, the sale of seal organs was the only activity with "excellent" market potential. This assessment is in agreement with an internal memorandum to Canada's Minister of Fisheries and Oceans, obtained through Access to Information legislation, which is even more emphatic in detailing the benefits of a harvest of seals for their penises: "...the demand, hence potential market, for seal sex organs has increased almost beyond comprehension. To be factual and frank, seals can now be harvested on a profitable basis solely for their sex organs; in fact, the entire TAC could be harvested on this basis I am told, and those involved (sealers and brokers) would make tremendous profit!" (Rideout 1993). Indeed, in the summer of 1993, a Chinese syndicate tried to buy 60,000 seals in Newfoundland and Labrador. It offered some native communities up to $50 (Can.) per seal, just for the penis and gall bladder (Lavigne 1994b). More recently, a company by the name of North American Environmental Technologies offered to buy and process 250,000 seals at a price of "twenty-five bucks for a large female seal, [and] double that for a male" (Canadian Broadcasting Corporation 1995; Lavigne 1995).

The fact that the most valuable part of the seal currently is its penis may influence hunters to kill preferentially mature males (potentially resulting in an adverse impact on the reproductive potential of the population). There is some evidence from the Canadian harp seal hunt, for example, that sealers may be beginning to target mature animals. Between 1972 and 1991, the mean percentage of "pups" (young of the year) taken in the annual Northwest Atlantic harp seal hunt was 80.7% (range of 60% to 91%). This percentage remained relatively constant for 20 years despite major changes in the seal hunt over this period: the imposition of the first Total Allowable Catch in 1971; Canada's 1977 extension of its economic zone to 200 miles, giving it exclusive control over its traditional sealing grounds; and the implementation of the European import ban on whitecoat harp seal pelts in 1983. In 1992 and 1993, data on the age structure of the seal harvest are not available, but for 1994 and 1995, there was a dramatic reduction in the percentage of pups taken in the hunt (35% and 53%, respectively) and a resulting increase in the percentage of older (1+) animals taken (ICES 1992; Department of Fisheries & Oceans, Canada 1994d, 1995). This change in the hunters' preference for older animals coincided with increased publicity over the lucrative nature of the penis trade in Asia (Rideout 1993; RT & Associates 1994).

Further evidence of a possible change in an existing hunt to maximize the harvest of penises can be found in Namibia, where the 1994 quota for Cape fur seals was 43,000 pups and 12,000 adult males (bulls). The former figure is slightly less than the 1993

quota of 48,000 pups, and the latter figure is the highest quota ever given for bulls, more than four times higher than the 1993 quota of 2,850 animals (Namibia 1994; Lavigne 1994a; Eliot 1995). Consistently, a confidential government report, leaked to the Namibian press, states that the bull quota "is not sustainable and this quota cannot be recommended for any scientific reason." (Namibian 1994). It goes on to say that "granting a quota of 12,000 bulls in 1994 could result in the complete collapse of the seals' breeding system and the population would need between 5 and 15 years (without any commercial harvest) to recover".

It has long been recognized that the biological characteristics of pinnipeds make them particularly vulnerable to the effects of commercial overexploitation. As Ehrenfeld (1970) noted, the "hypothetically most endangered" mammal is one with "valuable fur, hide, oil, etc., [which is] hunted for the market or hunted for sport" in the absence of "effective game management" (p. 129). This hypothetical mammal is also a predator possessing a long gestation period, low fecundity, reproducing in large aggregations, that has a restricted distribution but travels across international boundaries (Ehrenfeld 1970). Given that seals demonstrate most of the attributes that Ehrenfeld described, it is perhaps not surprising that seal populations, including those whose parts are currently in trade, have been depleted consistently by market hunting in the recent past (Busch 1985). In the southern hemisphere, for example, no fur seal population has yet recovered to the levels once shown by fur seal catches to have existed (Busch 1985, Croxall and Gentry

1987), and as recently as 1950 to 1970, commercial sealing reduced the Northwest Atlantic harp seal population off eastern Canada by one-half to two-thirds (Øritsland 1971).

Other economic and legal realities also suggest that an increased trade in pinniped parts has the potential to threaten seal populations worldwide. As Norse (1993) pointed out, "species that people use as commodities are inherently at risk of population reduction or elimination". Even the Summary Report of the latest forum on Canadian east coast seal management acknowledges that "There are no controls with regard to the organ market" and that "the demand for organs might increase which could lead to an increased seal take including illegal activity" (Department of Fisheries & Oceans, Canada 1995c). Indeed, an illegal take of seals specifically for their penises has already been documented. In 1994, for example, fishers on the Galapagos Islands illegally killed an undetermined number of Galapagos sea lions (*Zalophus californianus wollebaeki*), from a small population numbering some 30,000 (Trillmich 1979), and sent a consignment of their penises to Japan, "where they were to be tried out as a new aphrodisiac" (Pearce 1995, p. 29). The Japanese buyers reportedly paid US $50.00 for each penis (Pearce 1995). The large potential market for penises that currently exists in some Asian Pacific countries seems likely to grow in the near future as the populations of these countries continue to increase at the same time that large numbers of their citizens acquire more disposable income (Lavigne et al. In Press).

In the case of many seal populations, the problems of commercial exploitation are exacerbated by the lack of legal protection that currently exists for marine mammals. Unlike the United States, Canada, for example, has no Marine Mammal Protection Act and no federal Endangered Species Act. Canadian marine mammals are actually classified by Canadian law as fish (Department of Fisheries & Oceans, Canada 1991). Furthermore, Canada has successfully defeated all attempts to list most northern true seals, such as harp and hooded seals, on the Appendices of the Convention on International Trade in Endangered Species (CITES) (Herscovici 1985; Lavigne 1985). As a result, these species currently have absolutely no legal protection against the potential impacts of international trade.

To paraphrase Baker and Palumbi (1994), the "sustainable use" of seals is based on the assumption that only abundant species will be killed, that these hunts, and the subsequent trade in the hunted products, will be properly regulated, and that depleted species will continue to enjoy protection. The results of the present study suggest that, with respect to some of the world's seal hunts, the existence of a trade in pinniped penises may render these preconditions for sustainability impossible to fulfill.

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GENERAL DISCUSSION

Forensic science is constantly evolving in its technology and approaches to providing evidence. As the number of cases submitted to the McMaster laboratory increased, so has the number of requests to address the specific questions associated with a wildlife forensic investigation. Chapters 1 to 6 demonstrate the applications we have developed to provide evidence in the cases the McMaster laboratory has analysed. A second factor affecting the evolution of wildlife forensic science is the need to provide conservative unbiased evidence (Lander 1991) for the legal acceptance of DNA results. To achieve this criteria comprehensive DNA data bases are required for the DNA marker systems being applied to wildlife investigations. This section will present: a summary of the case work processed by the laboratory; the state of knowledge regarding the DNA data bases that have been established for the DNA profiling systems we have developed; the research and development of DNA profiling systems and DNA data bases; and an examination of the need for quality assurance guidelines in wildlife forensic science.

Summary of Wildlife Forensic Cases

The Ontario Ministry of Natural Resources (OMNR) has referred approximately 150 wildlife cases involving about 1000 samples over the past six years. Approximately 100 cases have required the individual identification of moose and white-tailed deer in cases involving poached animals. Approximately 50 cases have involved the species identification of animal tissues in illegal commercialization investigations. Several cases have required sex identification of game species to confirm validation tags. Two other provincial agencies have referred a large number of cases for analysis: since 1994 the Newfoundland Department of Natural Resources has submitted 25 cases involving DNA fingerprinting, species identification or the sex identification of moose samples. Between 1993 and 1995 the Alberta Fish and Wildlife Forensic Laboratory submitted over 25 cases for DNA fingerprinting analysis in the following cervid species: moose, mule deer, white-tailed deer, elk and caribou. The following agencies also submit cases for DNA testing: the Department of Natural Resources of New Brunswick, Nova Scotia, British Columbia, Manitoba and Saskatchewan. The Ontario Ministry of Agriculture and Food (OMAF) has also utilized the laboratory in cases of criminal fraud involving the substitution of beef samples submitted to the OMAF laboratory for inspection. We have demonstrated, using DNA fingerprinting, the submission of "clean" samples which did not originate from the animal tagged for inspection.

Federal Agencies have also utilized the McMaster laboratory. Parks Canada has submitted samples collected in poaching investigations from the following National Parks: Riding Mountain National Park, Manitoba; Kouchibouguac National Park, New Brunswick; and Gros Morne National Park, Newfoundland. The Canadian Wildlife Service, Atlantic Region submitted an investigation involving Common Eider ducks (*Somateria mollissima*) requiring parentage analysis to identify the presence of wild chicks in a captive breeding program. The RCMP from several regions have submitted cases for DNA testing including: DNA analyses to determine parentage in a case involving Peregrine falcons (*Falco peregrius*) suspected of being illegally obtained from wild stocks; a case of fraud involving Rottweiler dogs; and several cases involving poaching of white-tailed deer and moose.

The McMaster laboratory has been successful in presenting forensic evidence in provincial and federal courts. The following are examples of sentences passed down in cases for which DNA evidence was crucial in obtaining a conviction: $10,000 fine and 2 year hunting suspension in a case of unlawfully hunting moose; a total of $5,000 in fines for the illegal commercialization of black bear gall bladders; $36,000 fine and 10 year hunting suspension for unlawfully hunting moose (the North American record fine at the time of the conviction); $40,000 fine for unlawfully hunting approximately 40 white-tailed deer (the new record fine); and a 9 month jail sentence under the Federal Migratory

Bird Act in the Canadian Wildlife Service investigation of illegal possession of Common Eider ducks.

Established DNA Databases

The development of DNA data bases established the appropriate use of the DNA marker systems utilized in the profiling in an investigation. The basis for the genetic data bases is to demonstrate the level of DNA variation is consistent with the DNA marker being developed. Species-specific DNA markers require that the DNA variation is constant within a species but unambiguously different among species. DNA data from animals over a range of geographical regions in Canada was used to demonstrate this characteristic using satellite DNA markers (Chapter 2) thereby effectively satisfying this criteria for the species most commonly submitted for testing. DNA fingerprinting has proven to be sufficiently variable in a large number of species to allow unambiguous identification of individuals within a species. The focus of the DNA data base required for individual identification is to ensure a conservative estimate of the statistical probability of a match is generated.

Human databases have been established for single locus VNTR probes for use in forensic investigations and there has been considerable debate over the effect of population structuring in the presentation of DNA evidence (Cohen 1990; Chakaborty and Kidd 1991; Lander 1991; Lewontin and Hartl 1991; Weir 1992). General population data bases have been demonstrated to be sufficient for estimating the probability of a

match in human forensic cases as sub-division due to ethnic grouping or geographic location do not unfairly bias this estimation (Budowle et al. 1994). These data bases are based on allele frequencies obtained with single locus VNTR probes. The inability to assign allele frequencies to VNTR fragments with this approach requires the DNA data base to be based on the average level of band-sharing within a population. Population structuring is a factor which can increase this measure through inbreeding and genetic drift caused by isolation. Previous studies have used multilocus DNA fingerprinting to identify population structuring in wildlife species (Gilbert et al. 1990; Hoelzel and Dover 1991; Degnan 1993; Stacy et al. 1994). Identifying the appropriate populations for which to establish a DNA data base is of importance for determining a representative and conservative band-sharing coefficient for calculating the probability of a match.

An example demonstrating the need to consider population structuring in wildlife DNA data bases is evident in the moose data base established for eastern Ontario in a study addressing the genetic structure of this species. Band-sharing coefficients for pairs of moose representing several geographic regions in eastern Ontario had a mean band-sharing coefficient of 0.420 (Guglich et al. 1993, Chapter 1). Examining the mean band-sharing coefficients within and between the three main geographic regions of eastern Ontario (Swastika, Sault Ste. Marie and Pembroke) which contributed to the data base indicated a higher degree of band-sharing within regions than between regions (Table 1).

Table 1: Population Genetic Structure of Moose (*Alces alces*) in eastern Ontario. Mean band-sharing coefficients (D_{mean}) of within and between populations of moose using the methodology described in Guglich et al. (1993) for *AluI* digested DNA hybridized with the Jeffreys' 33.15 probe. Statistical comparisons of band-sharing coefficients calculated for within geographic regions to between geographic regions were calculated using the Mantel test (Mantel 1967) and 1000 permutations of the data in the NTSYS (Applied Biostatistics Inc.) (Rohlf 1990) system.

Within Geographic Regions

Geographic Region	Number of Moose	D_{mean}
Eastern Ontario	16	0.420
Swastika, Ontario	6	0.550
Pembroke, Ontario	5	0.517
Sault Ste. Marie, Ontario	5	0.493

Between Geographic Regions

Geographic Region	Number of Moose	D_{mean}
Swastika/Pembroke	11	0.384†
Swastika/Sault Ste. Marie	11	0.410*
Pembroke/Sault Ste. Marie	10	0.360†

Statistical comparison of within and between band-sharing coefficients using the Mantel test (1967):

*** $p < 0.050$**

† $p < 0.005$

A matrix of band-sharing coefficients was compared with a matrix of the distances, within and between the geographic regions, using the Mantel test (Mantel 1967). This approach has been used in demonstrating population structuring of root voles (*Microtus oeconomus*) from different geographic regions (Stacy et al. 1994). A significant difference between all three WMU indicated population structuring between the moose from these regions (Table 1). Low levels of dispersal between populations have been shown to homogenize within and between band-sharing coefficients (Keane et al. 1991).

Assuming the samples from eastern Ontario represented one population, the band-sharing coefficient used in calculating the probability of a match would not be representative of the actual genetic structure and thereby reduce the band-sharing coefficient as to bias against the suspected poacher. This effect is analogous to the Wahlund effect (Wahlund 1928) which underestimates the number of heterozygotes in a population as a result of sampling from structured populations with different allele frequencies. The majority of between region band-sharing coefficients are 0.100-0.200 lower than the within region band-sharing coefficients and more pairwise comparisons exist for between region comparisons, i.e. 85 between region pairwise comparisons compared to 35 within region comparisons, therefore the effect of pooling all the band-sharing coefficients will substantially decrease the observed mean band-sharing.

A conservative band-sharing coefficient of 0.600 is used in Ontario investigations for calculating the probability of a match which accounts for population sub-structuring

in moose (Guglich et al 1993, Chapter 1). This value is higher than the mean band-sharing coefficients for the three main geographic regions (Table 1) and therefore maintains the criteria of conservative in a legal application. The probability of a match using the mean band-sharing coefficient of 0.420 is approximately 7500 times higher the than estimate which accounted for population structuring (0.600). The two band-sharing coefficients both provide convincing probabilities of a match between samples, however the approach must be conservative and therefore population structuring must be considered in establishing a DNA fingerprint data base for calculating representative statistics. We have established DNA fingerprinting data bases of isolated moose populations such as Lake Abitibi in Ontario, Riding Mountain National Park in Manitoba and the island populations of Isle Royale, Michigan and Newfoundland which may have undergone small founder events (Wilson and White in preparation).

The DNA data base we established from the population of white-tailed deer sampled from Manitoulin Island demonstrated similar levels of band-sharing to southern Ontario samples (Guglich et al. 1993). A conservative estimate of 0.50 is used in the calculation of a match in deer poaching cases and this value appears sufficiently high to account for population structuring in this species. Samples collected from additional populations of potentially "isolated" populations, Pinery Provincial Park and Long Point National Park, have been submitted for analysis to expand the data base and confirm the

Chapter 6

Mitochondrial DNA extracted from eastern North American wolves killed in the 1800s is not of grey wolf origin.

The application of PCR technology to genotype microsatellite loci for individual identification is presently being established in our laboratory. We have identified more than 20 microsatellite primer sets isolated from bovine and cervids of which a substantial number of primer sets demonstrate cross-homology with other game species. For example, eight bovine (Research Genetics Inc.) and three white-tailed deer primer sets amplify polymorphic loci in moose. Our preliminary data indicates approximately 50% of ungulate microsatellite primers amplify polymorphic loci in other ungulate species and this cross-homology has been observed in other surveys of ungulate species (Engel et al. 1996). The selection of primer sets for moose, white-tailed deer, elk and caribou will be followed by: an accurate determination of allele sizes and the development of strict scoring criteria; the optimization of multiplex reaction conditions to simultaneously amplify several loci when possible; and the establishment of reference population data bases.

Species Identification

As outlined in Chapters 5 and 6 the most useful profiles for animal species identification using PCR appear to be sequences of the mitochondrial cytochrome b gene and the control region. The data base requirements for these markers are mitochondrial sequences from the species of interest, sequences from closely related species of the species of interest and additional species associated in investigations which are required to provide an exclusion from the species a suspect will claim is the source of a sample. As

more exotic species involved in the illegal trade of animal parts are being submitted to the laboratory the species DNA data base must be expanded and modified. A limitation which was encountered in the seal penis study in constructing a seal data base (Chapter 6) was the need to utilize EMBL/Genbank sequences due to a lack of the appropriate control tissue samples. EMBL and Genbank do not require continuity procedures and the source of the sequence is not confirmed upon submission making these sequences unacceptable for use in forensic data bases. Continuity and documentation of data base samples are required for the collection and verification of the samples and maintenance of security of the data base information is required once it is completed. A typed specimen represents a "gold standard" for a species and must meet the following guidelines prior to the acceptance of a sample into the data base: positive identification of the animal by an expert qualified in identifying the species based on morphological characteristics; photo documentation of the animal accompanied by sample information, i.e. genus, species, sub-species, geographic origin, ect., and written verification by the qualified expert; and maintenance of the continuity of evidence as it applied to case samples (Woodley, in press).

Population Identification

Animal populations which require specific markers for identification can be assessed by the mitochondrial control region (Baker and Palumbi 1994) or using genomic markers such as microsatellites. One of the immediate issues in Ontario requiring

population identification is the illegal sale of bear gall bladders. Gall bladders obtained within a province can not be legally sold in that province, but it is not illegal to sell or purchase galls obtained from other provinces. Samples of confiscated bear gall bladders are presently being collected from regions throughout Canada by Alan G. Sullivan, OMNR Intelligence Specialist, and DNA markers obtained from these tissues will be incorporated into the data bases.

Research and Development: Quality Assurance

Wildlife DNA forensics laboratories may have to soon meet the same standards as human forensic laboratories with respect to planning and organization of laboratories, personnel, documentation, validation, equipment, materials and facilities, evidence handling procedures, security, analytical procedures, case work, documentation, interpretation, report writing and review, proficiency testing, audits and safety. The Technical Working Group on DNA Analysis Methods (TWGDAM) was formed in November 1988. The purpose of TWGDAM is to provide a forum for discussing issues and reaching a consensus on methodologies and their publications are considered as guidelines to assist the individual crime laboratories in the establishment of their DNA programs. Woodley (in press), based on the TWGDAM working group reports, has provided a range of recommendations that would apply to wildlife DNA forensic laboratories.

Ms. Christine McLaren, a fourth year honours student, conducted a review of quality assurance guidelands proposed for human forensic DNA analysis to recommend a similar set of guidelines for wildlife forensic science. Ms. McLaren reviewed the TWGDAM reports and the standards of additional working groups, i.e. American Society for Histocompatibility and Immunogenetics and American Association of Blood Banks, and complied the results of surveys completed by forensic laboratories in Canada, the U.S. and the U.K. A second aspect of the project was to review the McMaster laboratory: "It can be concluded that despite a lack of established quality assurance guidelines, evidence sample collection, continuity of evidence, the analytical procedures and data interpretation performed by the (McMaster) laboratory staff are excellent." (McLaren 1996). Recommendations for improvement included the McMaster laboratory and the Canadian wildlife forensic laboratory community. Within the McMaster laboratory the use of standard Chain of Custody documents for each case was advised. Recording the continuity of evidence has been standard practice in the laboratory and since this recommendation forms have been developed and implemented into the forensic service to standardize these records. Proficiency testing of laboratory personnel and the establishment of quality assurance were highly recommended for Canadian forensic laboratories.

Ms. Stephanie Pendry, an M.Sc. student from the University of Kent at Canterbury, surveyed the laboratories in Canada that were providing wildlife DNA

forensic service in 1993 and in 1995-96 Ms. C. McLaren (McMaster University) updated the survey. This survey indicated that there were five Canadian laboratories: the McMaster laboratory; the Bio-ID laboratory owned and operated by Dr. W. Davidson at Memorial University in Newfoundland, known for the species-identification service using FINS (forensically informative nucleotide sequences)(Bartlett and Davidson 1994); the Quebec Ministry of Natural Resources has established a laboratory in Quebec City; in Alberta Mr. R. McClymont of the Alberta Ministry of Natural Resources in collaboration with Dr. Curtis Strobeck at the University of Alberta have initiated a DNA service in conjunction with the existing protein forensic service; in British Columbia the Helix Corporation were initiating a small service to complement their human profiling service; and a crown corporation, the Research Productivity Council (RPC) in New Brunswick directed by Dr. Steve Griffiths has initiated a DNA service. Much of the DNA forensic work in the U.S. is handled by the U.S. Fish and Wildlife Service in Oregon, although the number of state operated wildlife forensic laboratories are increasing.

There has been communication and co-operation among several of the Canadian laboratories. The Alberta and New Brunswick laboratories used the McMaster laboratory while they established DNA procedures and the three laboratories have exchanged technicial information and information on DNA data bases. Establishing external proficiency testing as part of a quality assurance (QA) mechanism would help ensure common standards. On a periodic basis Canadian laboratories or government

agencies could provide "mock" cases to the other laboratories. The demand for a Canadian network of wildlife forensic science is warranted and the expertise now exists to establish the appropriate standards and communicate the technology among laboratories through annual workshops.

Conclusion

DNA profiling has proved to be an effective tool in providing evidence in wildlife forensic investigations and this evidence is intrinsically important to each case, however, it is difficult to accurately assess the effectiveness of applying DNA technology as a deterent to poachers. Two factors are required to establish an effective deterent for poachers: a high expectation of being charged and convicted; and that high penalties be issued and enforced by the courts. The estimates of apprehending poachers is low, approximately 1%, and fines that are issued upon conviction are also low (Gregorich 1992). There are notable exceptions to both these findings with respect to higher conviction rates and higher fines imposed in cases involving DNA evidence (see examples of penalties in the Summary of Wildlife Forensic Cases).

DNA evidence may act as a deterent through the press coverage of the wildlife cases. DNA forensic science has attracted media attention and the role of the McMaster laboratory in wildlife cases has been covered in local papers, the Globe and Mail and interviews on CBC radio and television. Press coverage in local news may attach a stigma

to poaching making it less acceptable in rural communities and publicity generates the message that DNA technology has increased the probability of a poacher being charged and convicted. DNA technology may also influence the fines issued to convicted poachers. A presiding judge or justice-of-the-peace (J.P.) may be influenced by the technology that has been introduced in wildlife investigations. DNA evidence has had a major impact on human forensic science and the presentation of similar evidence to court officials in wildlife cases may generate the perception of high-technology, high cost and a serious committment of conservation officers and the Ministry of Natural Resources to the apprehension and conviction of poachers. Judge Mahaffy's quote demonstrates the impact DNA evidence can have on the presiding judge (see General Introduction). These two deterents are likely to be a more effective deterent for local poachers in communities than for "professional" poachers involved in the exploitation of wildlife for profit.

Illegal commercialization of game meat and the illegal trade of animal parts require deterents that decrease the cost-effectiveness of the illegal activity. "As long as there is a market for wildlife, there will be vendors; where there are vendors, there will be suppliers, and where there are suppliers, there will be harvesters." (Gregorich 1992). In illegal commercialization infractions, DNA technology will impact as a deterent most effectively on "legitimate" businesses (vendors), i.e. butcher shops, that may suffer from the press coverage of illegally selling game meat. In these cases the probability of a charge and conviction are high based on the ability of conservation officers and food inspectors to

readily obtain samples for testing. Any deterents to black market vendors or suppliers must include high penalties. In investigations of this nature, DNA testing as a deterent is proportional to the severity of the penalties. In some regions a fine is incorporated into the "cost-of-doing-business". The illegal trade of animal parts is often associated with organized crime and other illegal activities such as drug and gun smuggling (Gregorich 1992). As an example, Asian gang members in southern Ontario have been implicated in illegal fishing and the trade of animal parts, but small fines are insignificant to a group involved in credit card fraud and other profitable crimes.

Therefore, establishing effective deterents to poaching and the illegal trade of animal parts requires the manpower to investigate the infractions, forensic technology to provide convincing evidence to achieve a conviction, an educated public and court system that recognizes the gravity of wildlife infractions and the legislation to issue penalties that outweigh the benefits of poaching and trafficking wildlife.

The McMaster laboratory has provided effective enforcement tools for providing conclusive evidence in wildlife forensic cases. Research and development will continue in the laboratory to provide the neccessary testing required by the governement agencies protecting the natural resources. Education is also a priority within the laboratory: a web site home page has been constructed (http://www.science.mcmaster.ca/Biology/ services/forensic) by Ms. McLaren; and a multimedia computer package has been assembled in collaboration with Mr. Steve Harrington and Mr. Jose Castillo, formely of

Sheridan College. Both packages are designed to educate at different levels of interest, i.e. school children, forensic scientists, conservation officers, lawyers and judges, in an attempt to transfer technical information and communicate the importance of protecting our natural resources.

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