P.J. Wilson,[1,2] *M.Sc. and B.N. White,*[2] *Ph.D.*

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 technique 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: forensic science, polymerase chain reaction, Sry, Fmr-1, elk, moose, white-tailed deer, sex identification, wildlife, DNA typing

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 (4–6). 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: first, the amplification of two homologous loci, one on the Y-chromosome and the other on the X-chromosome, that demonstrate chromosome-specific differences, such as the presence or absence of a restriction enzyme site (4,7); and second, a multiplex reaction which utilizes two primer sets to amplify both the diagnostic region of interest, such as 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 of elk, moose, and white-tailed deer using Sry primers based on ungulate species, that is, sheep and cattle (9), and the human Fragile X gene sequence (10) to act as an internal control.

[1]Department of Biology, McMaster University, Hamilton, Ontario, Canada.
[2]Wildlife Forensic DNA Library, Trent University, Peterborough, Ontario K9J 7B8

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Materials and Method

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 morphological 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 to 24 h. 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 2× volume of 95% ethanol. Precipitates were centrifuged at 3000 × g for 15 min, washed with 70% ethanol and recentrifuged. The DNA was dissolved in 1× 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 by 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′

The SRY primers correspond to the nucleotide positions 2–20 and 58–78 from a published bovine Sry sequence (9). Reactions were performed in a total volume of 50 μL under the following conditions: 10 mM Tris-HCl pH 8.4, 50 mM KCl, 0.001% Triton X-100, 1.5 to 2.0 mM $MgCl_2$ and 1 U of Taq polymerase (Perkin Elmer Cetus). Amplification was performed under a temperature regime of: 94°C for 5 min, 55 to 60°C for 30 s, and 72°C for 30 s for 1 cycle; 94°C for 15 s, 55 to 60°C for 30 s and 72°C for 30 s for 30 cycles; and 94°C for 15 s, 55 to 60°C for 30 s and 72°C for 5 min 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 of 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 without modification. The temperature regime was as described above using a 60°C annealing temperature. Both mixed and nonmixed samples were processed simultaneously.

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

Cervid Species	$MgCl_2$ Concentration	Taq Polymerase (units)	Annealing 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 virginianus	2.0 mM	1.0 U	60°C

Electrophoresis

PCR products were analyzed by gel electrophoresis on agarose gels. The amplified DNA was electrophoresed through 1.0% agarose gels at 80 V for 3 to 4 h in Tris-borate buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Flanking molecular weight size standards of 123 bp ladder (BRL) were run on each gel. The PCR products were stained with ethidium bromide and visualized under ultraviolet (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 reamplified 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 were 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.). 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 dlTTP, 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 180 bp Sry fragment and an Fmr-1 product of approximately 350 bp 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 nonspecific 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 nonspecific 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 (Fig. 3). The sequence homology among the three North American cervids and the bovine Sry gene sequence was calculated at 96.4 to 98.6%. The sequence homology between

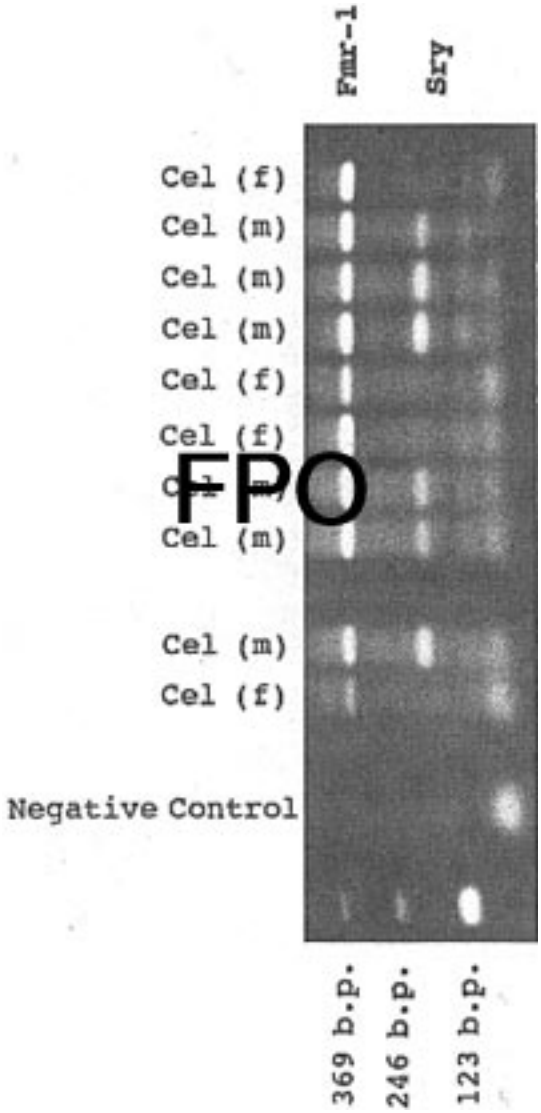

FIG. 1—*Ethidium stained agarose gel (1.2%) of multiplex PCR products from elk (*Cervus elaphus*, Cel) male (m) and female (f) DNA. The Sry and Fmr-1 products are identified. Primer-dimer artefacts can be visualized in the negative control. Female samples demonstrate the Fmr-1 product alone and the male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

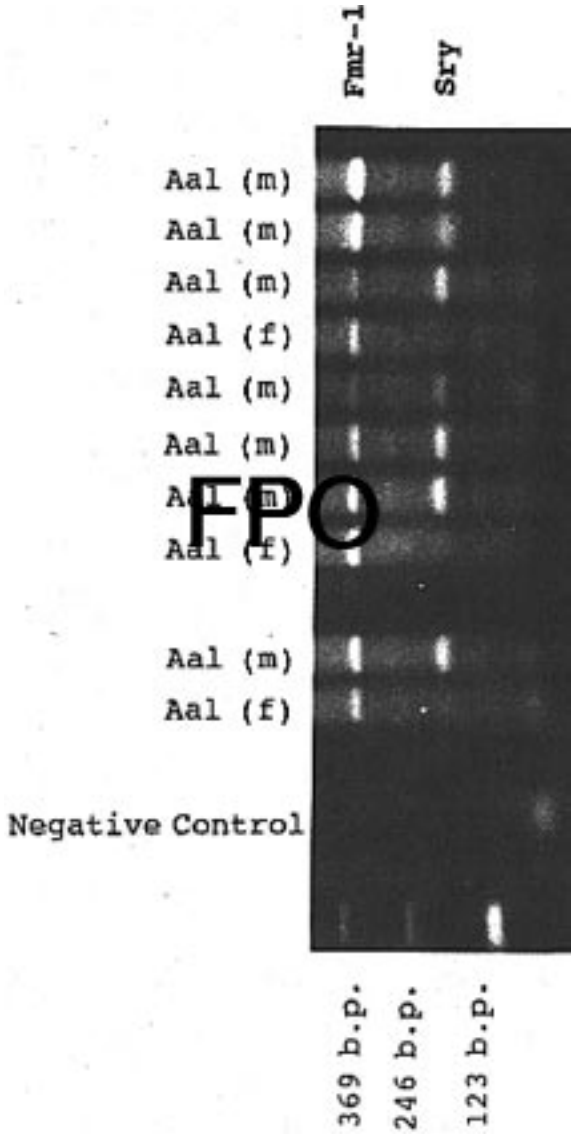

FIG. 2—*Ethidium stained agarose gel (1.2%) of multiplex PCR products from moose (*Alces alces, *Aal) male (m) and female (f) DNA. The Sry and Fmr-1 products are identified. Primer-dimer artefacts can be visualized in the negative control. Female samples demonstrate the Fmr-1 product alone and the male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

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Elk              5′ tgaacgaagacgaaaggtggctctagagaatcccgaaatg
White-tailed Deer   ----------------------------------a-----
Asian sp.           ----------------------------------a-----
Moose               ----------------------------------c-----
Bovine              ----------------------------------a-----

caaaactcagagatcagcaagcagctggggtatgagtggaaaaggcttacagatgctgaaa
------------------------------------------------------------
-------------------------------------------c----------------
----------c-------------------------------------------------
----------c-----------------a--------------------------------

agcgaccattctttgaggaggcacagagactactagcc 3′
----c-------------------------------
----c--g-----------------------------
---------------------------------ga--
----c---------------------------gtag-
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FIG. 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.*

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 bp, 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 has been associated with the Fragile X syndrome (10). No 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 had been 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 unmodified 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.

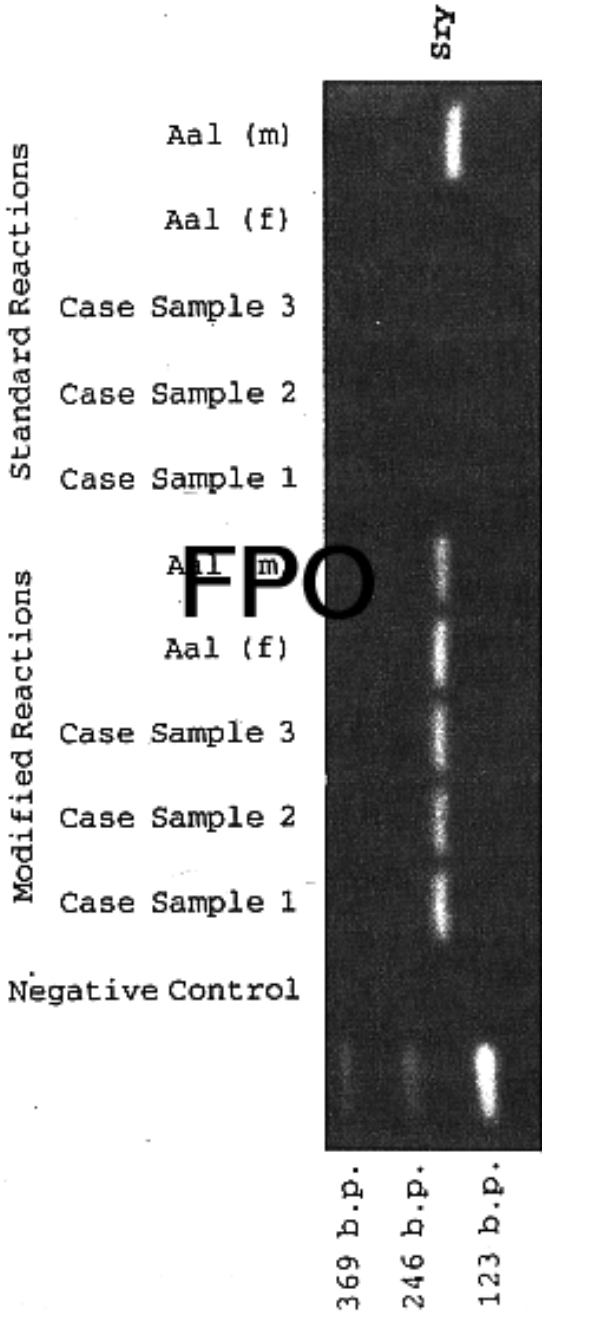

FIG. 4—*Confirmation of the multiplex results in the wildlife investigation utilized the Sry primers in identical reaction mixtures to amplify the template DNA of the case samples in one reaction set (Standard Reactions) and amplify the template DNA with added male moose DNA in the second reaction (Modified 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.*

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 (Figs. 1 and 2). 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) which demonstrates 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, that is, 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 amplification 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 with the Sry product, which is potentially important in severely degraded DNA samples. A degraded DNA sample which contains fragments of less than 350 bp 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 that 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 of 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 with 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 eliminate the issue of false-positives being generated due to DNA contamination from human sources. The specificity 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 because 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 analyzing 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 courtroom. 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 multiplex 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 deterrent against hunters violating the validation system.

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Additional information and reprint requests:
P.J. Wilson
Department of Biology, McMaster University
1280 Main Street West
Hamilton, Ontario L8S 4K1