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A simple and improved PCR-based technique for white-tailed deer (*Odocoileus virginianus*) sex identification

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Abstract We describe a simple single-reaction technique for identifying the sex of white-tailed deer (*Odocoileus virginianus*) based on the PCR amplification of a zinc-finger intron using one pair of primers. Although *Sry*-coamplification confirmed sex identities, use of the *Sry* marker was unnecessary due to dimorphic alleles on the X and Y chromosomes at the zinc-finger locus. Insertions in intron 7 of the Y-linked allele (417 bp) make it nearly twice as long as the X-linked allele (236 bp) and thus the amplification products are easily discernable by simple agarose gel electrophoresis. The relatively short size of these products makes them useful for DNA-based sex identification from potentially low-yield tissue samples (e.g., hair, feces). This technique will provide ecologists, conservation geneticists and wildlife managers with a mechanism to readily and reliably identify the sex of unknown white-tailed deer tissue samples, and likely similar samples from other cervid species.

Keywords ZFX/ZFY · Sex-linkage · Cervidae · Wildlife management · *Sry*

Introduction

Recent advances in molecular techniques and non-invasive sampling protocols provide biologists with new approaches for estimating species prevalence, distribution, and abundance across large geographic areas (Mowat and Strobeck 2000; Woods et al. 1999). Genetic markers suitable for species, sex, or individual identification have now been developed for many wild mammalian species (Foran et al. 1997a, 1997b; Morin et al. 2005; Villesen and Fredsted 2006). Identification of wildlife species from tissue samples has direct application to conservation assessments, including the monitoring of wildlife harvests. Many worldwide cervid species (Mammalia: Cervidae) are hunted and collected for food, skins and trophies. Cervid harvest regulations set by governing bodies usually regulate harvest in a sex-specific manner—that is, they allow differential harvest of male and female animals. In North America, this is typified by regulations established for the harvest of white-tailed deer (*Odocoileus virginianus*) (Riley et al. 2003). To facilitate compliance with applicable laws and to reduce poaching (e.g., Gilson et al. 1998), techniques that correctly, efficiently and dependably identify cervid sex are needed for conservation law enforcement. Further, reliable technique for determining sex of these mammals will aid ecological, evolutionary and conservation-oriented studies that require sex-identification data from indirectly or passively collected samples.

There have been several methods previously described for molecularly identifying the sex of mammals, including cervids (Bryja and Konecny 2003; Carmichael et al. 2005; Morin et al. 2005; Shaw et al. 2003; Wilson and White 1998). These protocols typically use an assay that differentiates sexes based on attempted PCR am-

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plification of a Y-linked (e.g., *Sry*) gene. However, the lack of an *Sry* amplification product may not indicate the lack of a Y-chromosome (Taberlet et al. 1993) but instead a lack of PCR effectiveness. Consequently, some methods incorporate an internal positive control for the *Sry* PCR reaction by coamplifying an X-linked gene (e.g., *amelogenin* or the Fragile X locus; Taberlet et al. 1993; Wilson and White 1998) often in a single-tube multiplex reaction with primers for both the X-linked and Y-linked loci. Another approach is to identify single nucleotide polymorphisms (SNPs) in alleles found on both the X and Y chromosomes (X and Y alleles are ‘gametologs’ rather than homologs: *sensu* Garcia-Moreno and Mindell 2000), where one allele contains a restriction site around the SNP and the other does not. PCR amplification followed by restriction digestion can then be used to differentiate XX individuals from XY individuals (Fernando and Melnick 2001).

Optimally sex-identification should be accomplished with a PCR-based method that amplifies a locus with differentiable alleles found on both the X and Y chromosomes, rather than amplifying different genes from each. However, finding copies of genes on both the X and Y chromosomes is difficult given the evolutionary loss of genetic material from the Y-chromosome (Vallender and Lahn 2004). The lack of recombination between mammalian X and Y chromosomes leads to different evolutionary histories and this divergence can putatively create alleles with significant size differences. Amplifications of different sized gametologs could then be used in an electrophoretic sex-identifying assay.

This process has been developed for cetaceans (Morin et al. 2005) and primates (Villesen and Fredsted 2006), although the described differences between gametologs in these taxa are either single base-pair substitutions or small (<30 bp) indels—difficult polymorphisms to differentiate without specialized (and expensive) lab equipment and supplies. For white-tailed deer, Shaw et al. (2003) described a 50 bp deletion in the Y chromosome allele found in a 930 bp intron of the zinc-finger gene. Although amplification of this marker produces adequate band separation on an agarose gel, such a large fragment requires a DNA sample of relatively high quality for reliable and dependable amplification. High quality DNA is not always available to wildlife managers who may be working with limited samples of DNA. Our goal was to design a PCR-based protocol that would return reliable sex-identification data to scientists with limited resources or expertise and who are often working with degraded and/or small quantities of tissue.

Materials and methods

Samples

We analyzed tissue samples from deer of known sex harvested from two populations of the USA: Wisconsin (hair samples) and New Hampshire (skin samples). Hair samples were collected at deer registration stations in Bayfield County, Wisconsin, from white-tailed deer harvested by hunters during November 2005. Before hair samples were collected, the sex of individual specimens was determined through observation of external genitalia. Collected samples were placed in individually-labeled coin envelopes and allowed to air dry until transported to the laboratory. A minimum of 10 guard hairs with follicular material were collected from each deer. Skin samples were obtained from New Hampshire Department of Fish and Game, where tissue samples were taken from animals opportunistically collected as part of a routine chronic-wasting disease monitoring program. These deer were also sexed before skin samples were collected. Small skin samples were cut from the neck or back of the head and then placed in individually-labeled plastic bags where they remained frozen until analysis. We randomly selected five male and five female samples from each population to test these procedures.

DNA extraction and PCR Amplification

We extracted DNA from deer hair and skin samples with Qiagen DNeasy Tissue Kits (Qiagen Inc., Valencia CA) according to manufacturer’s protocols. PCR reactions were performed with Platinum® *Taq* DNA polymerase (Invitrogen) using approximately 25 ng of DNA template with adjustments made to the manufacturer’s recommended reaction protocol to account for quarter-reaction volumes (12.5 μ L). For reactions amplifying the zinc-finger intron, we designed primers based on published *Capreolus* spp. sequences: CerZFXyf (5′-GCTGACCCCTGGAGAAGATGACTTA-3′) and CerZFXyr (5′-TCATTCTCAGGCTCACTCTCCACA-3′). Primers for amplifying the *Sry* region were designed from conserved *Sry* sequences of several published Cervid sequences: CerSRYf (5′-TGAACGAAGACGAAAGGTGGCTCT-3′) and CerSRYr (5′-TACCCTATTGTGGCCAGGTTTGT-3′). We ran separate reactions with each set of primers, as well as a duplex-PCR reaction that included both sets of primers. For each set of reactions, the thermal profile began with an initial 3-min 94°C denaturing step, followed by 35 cycles of: denaturing at 94°C for 30 s, primer-annealing at 55°C for 30 s and

fragment elongation at 72°C for 60 s. The cycles were followed by a final 5-min elongation step at 72°C and reactions were then stored at 4°C before electrophoresis and visualization. Amplification products were visualized using UV light on ethidium bromide-stained 1.5% low-melt Tris-Borate-EDTA agarose gels.

DNA sequencing

To verify the identity of products amplified in the PCR reactions, we ran 50 μ L reactions for both sets of primers using two male (BAY018 and BAY019) and two female (BAY003 and BAY011) DNA samples, and then excised each band from the gel using a clean scalpel. PCR products were extracted from the agarose slices using Qiagen QIAquick® Gel Extraction Kit (Valencia, CA) per the manufacturer's instructions and then used as templates for sequencing reactions. Single-stranded cycle sequencing reactions were run for both strands of each product in 11 μ L volumes using Big Dye® Terminator v3.1 Cycle Sequencing Kit. Excess primers and nucleotides were cleaned with Sephadex columns (Princeton Separations), dried and re-suspended in 20 μ L of deionized formamide and run on an ABI Prism® 3100-Avant Genetic Analyzer. Primers were trimmed from aligned sequence chromatograms of the forward and reverse strands of each PCR product and discrepancies between base calls of both strands were reconciled visually.

Results

PCR amplifications

All five male and all five female samples from both hair and skin tissues had identical PCR profiles (within each sex). From multiplex (Fig. 1A) PCR reactions that included both zinc-finger and *Sry* primers, females showed a single band and males showed three bands. Amplifications with the *Sry* primers alone (Fig. 1B) showed no amplification products from female samples but obvious products from the male samples (~350 bp in length). Amplifications from the zinc-finger primers alone (Fig. 1A) showed dimorphic results for male and female samples. Both sexes showed a single band representing a product ~220 bp in length and males showed an additional band representing a product ~410 bp in length.

Sequences

Sequences obtained from the *Sry* locus amplification were 361 nucleotides long and identical between the two individuals sampled (Bay018 and Bay019). The *Sry* sequence

was AT-rich (52.63%: considerably adenine biased 34%) and contained an open reading frame throughout its length. This sequence (Genbank accession #EF077163) differed at three positions from a portion (positions 222–582) of an *Sry* sequence of *Rangifer tarandus* and seven positions from same genomic segment of *Alces alces*. At the zinc-finger locus, we retrieved identical AT-rich (64.83%) sequences from the smaller fragments (see Fig. 1B: 236 nucleotides long) amplified from females and males (all four products were sequenced). This sequence (Genbank accession # EF077164) aligns with intron 7 and a small portion of exon 8 of the X-linked zinc-finger protein coding sequence of several cervid species, as well as *Bos taurus*. Nucleotides from this fragment differed with other published cervid zinc-finger protein sequences at five positions, and from *Bos taurus* at eight positions. The longer band from the male zinc-finger amplification (Fig. 1B) was 417 bp in length and the sequence (Genbank accession #EF077165) was closely aligned with intron 7 of the *Bos taurus* ZFY sequence (40 mismatched including two gaps that total 15 nucleotides in length). This sequence showed similarity to sections of the smaller X-linked product as well, with the largest sections of putative homology appearing near the end of the fragment closest to the reverse primer, while the other end of the fragment had at least one and likely two large (>20 bp) insertions on the Y-linked allele.

Discussion

This technique provides a sex-identification protocol for white-tailed deer that amplifies a single locus with alleles on the X and Y chromosome that differ in length by 189 bp, where the Y-linked fragment is nearly twice as long as the X-linked fragment. Fragments used in this protocol are considerably shorter than those previously described for the zinc finger gene in cervids (Shaw et al. 2003). This technique does not require co-amplification of multiple loci (Wilson and White 1998) nor real-time PCR capabilities (Morin et al. 2005). Conveniently, the size difference between these gametologs is large enough to be discerned under even minimally stringent conditions, and the products were amplifiable from tissue samples as potentially unreliable as hair. A brief word of caution is worth note regarding the longer Y-linked allele. Since highly degraded DNA can result in short modal fragment sizes (<250 bp), the X-specific gametolog may be less susceptible to degradation than the Y-specific gametolog. As a result, researchers using tissues that are likely to suffer from high levels of degradation should be wary of mis-identifying males samples as coming from female individuals.

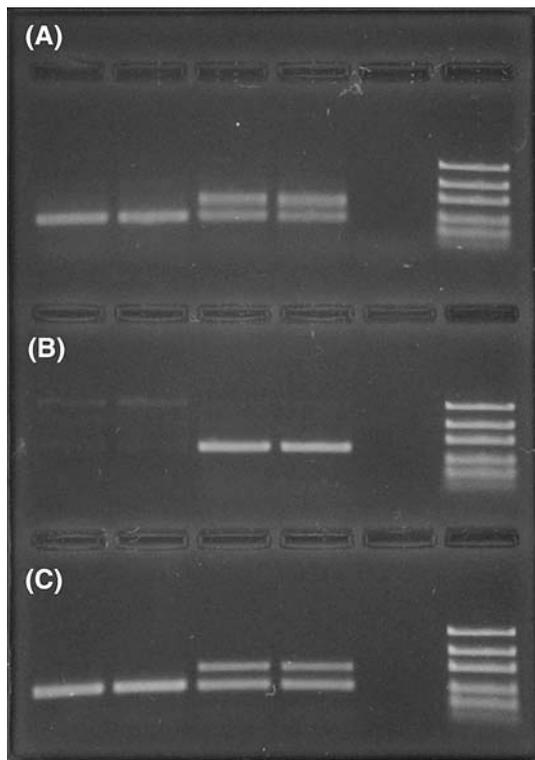


Fig. 1 Sample gel image generated from PCR amplification of male and female white-tailed deer sex-linked genes. (A) multiplex PCR results using both zinc-finger and *Sry* primers, (B) *Sry* amplification alone, (C) zinc-finger amplification alone. In each row, lanes 1 and 2 are female samples, lanes 3 and 4 are male samples, lane 5 is the negative control and lane 6 is the ladder (1, 2, 3, 5, 7, and 900 bp)

Shaw et al. (2003) described a molecular method for determining the sex of several mammalian taxa, but the size difference between the X and Y alleles was much smaller (only 50 bp) than the difference described here, especially when compared to the overall fragment length (i.e. 880 bp and 930 bp fragments versus 236 bp and 417 bp fragments). It is also notable that from our amplifications the Y allele was longer than the X allele (similar to the homologues found in *Bos taurus*), whereas the Y allele was shorter in the Shaw et al. study. Unfortunately, Shaw et al. (2003) did not publish the sequence of their fragment, nor did they identify which introns or exons were amplified by their technique. Cathey et al. (1998) originally published the primers used in the Shaw et al. study, but also did not identify the sequence of the introns or exons amplified.

Development of this technique has several important implications for wildlife management. From a population demographic perspective, use of this technique with non-invasive sampling methods can allow investigators to estimate sex ratios, monitor trends in abundance, and evaluate sex-mediated gene flow within and across deer

populations (Paetkau et al. 1998). Additionally, with considerable testing and vetting with greatly extended sample sizes, this simple protocol could have direct application to wildlife forensics and law enforcement. For example, the ability to effectively enforce game laws would be enhanced through application of this simple and effective technique to small tissue samples (e.g., hair) commonly used as wildlife evidence (Gilson et al. 1998). The simplicity of the technique means even basic molecular genetic laboratories could perform this test with great resolution and reliability.

Conclusions and future directions

This technique has been developed for sexing white-tailed deer, but it would likely prove useful for sexing other cervid species, and perhaps other artiodactyls, given the cross-species application of similar zinc-finger based techniques (Morin et al. 2005; Shaw et al. 2003; Wilson and White 1998). Although previous studies have provided more complicated protocols to distinguish sexes of white-tailed deer tissue samples, this technique provides several advantages for researchers: (1) small tissue samples with degraded DNA (like hair or feces) can be readily used to assay the sex of an individual animal, (2) a single set of primers is able to amplify a sexually dimorphic product, (3) the resulting amplification products are significantly different in size, which allows unambiguous scoring, and (4) the single PCR reaction (using 0.25 unit of Taq) followed by simple agarose gel electrophoresis is approachable by laboratories with the most basic of equipment.

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