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Genetic protocols for DNA extraction from white-tailed deer cast antlers to confirm individuality

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Abstract

White-tailed deer (*Odocoileus virginianus*) are the most sought-after deer species in America. The antlers of mammals, such as deer, are one of the fastest regenerative tissues in the world and are grown and naturally cast every year. Research on cast antlers have been used for a variety of purposes including population comparisons and impacts of deer health due to climatic stressors. When investigating cast antlers, it is important to confirm individuality of match sets in addition to antlers of the same individual between years. Therefore, individuality must be confirmed genetically, and protocols must be developed and established to do so. Our objectives were to 1) establish a genetic protocol to harvest DNA from cast antlers using connective tissue, and 2) determine individuality from subsequent years and match sets. When fresh antlers are cast, they leave behind a viable connective tissue from which DNA can be extracted. The DNA was successfully extracted from the skin rings harvested from naturally cast antlers. This study developed viable methods to confirm individuality, which aid researchers and wildlife biologists in a better understanding of the white-tailed deer herd as they set management goals and harvest regulations.

Keywords: Cervidae, heteroplasmy, Nebraska, *Odocoileus virginianus*, Platte River, shed antler cast, white-tailed deer

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Introduction

Antlers are paired appendages that are grown and cast annually by most deer species. Naturally cast antlers present a non-invasive method to study antler growth, deer health, and impacts of environmental pressures. Long term cast antler metric investigations on white-tailed deer (*Odocoileus virginianus*) have compared populations and management strategies (Ditchkoff et al. 2000), selective harvest (Schoenebeck and Peterson 2014), and the impacts of environmental stressors on deer health (Peterson et al. 2019). Without a method of genetic confirmation, cast antler match sets are often assumed based on physical proximity to each other in addition to antler similarities (i.e., size, burring, and coloration). For example, individuality was assumed if antlers were collected close together (<4.5 m), while antlers were assumed to be unique individuals at greater distances (Michael 1965). To avoid pseudoreplication of a match set, some studies only definitively differentiate an individual's cast antlers if the herd was high-fenced, closely monitored, photographed, or observed casting (Fierro et al. 2002, Landete-Castillejos et al. 2010, Moyes et al. 2011). In most free ranging cervid studies, only one cast antler side was used, e.g., left, or right (Ditchkoff et al. 2000, Landete-Castillejos et al. 2013, Peláez et al. 2018) or the first antler collected (Schoenebeck and Peterson 2014, Peterson et al. 2019). Indeed, genetic conformation of individuality would allow cast antler studies to avoid these assumptions.

Currently, at the University of Nebraska at Kearney, studies are developing predictive antler metrics to define a cast antler match set, therefore, genetic confirmation is critical to confirming individuality of match sets in addition to antlers of the same individual between years. In large mammals, DNA can be extracted from a multitude of tissues, both invasively and non-invasively. Soft tissues are utilized from deer to analyze DNA at check stations for disease or to develop a genetic lineage of the species in question. Soft tissue DNA extraction involves directly interacting with the animal to retrieve the source for extraction. Cast antlers provide a unique indirect and less intrusive source of DNA, and unlike soft tissues, the DNA degrades much more slowly. Environmental conditions affect weathering rates of antler and bone, and their

utility as DNA sources. An extraction method to retrieve DNA from weathered antler includes using an 8mm bit and drilling 2-3 cm deep to collect the material. Using this method, it was concluded that out of 107 antlers, 96 yielded pure DNA (Lopez and Beier 2012). Museum pieces have been used to extract antler DNA (mechanical drilling) to investigate the family history of the species. DNA was successfully extracted from all antlers in moderate to high concentrations, showing antlers do contain reliable DNA (Hoffmann and Griebeler 2013). Antlers have shown viable DNA up to 200 years old and other boney tissues have reached >50,000-year-old bone (Lopez and Beier 2012, Hoffmann and Griebeler 2013). Currently, there are no published studies showing the utility of using genetic approaches to match hypothesized paired antlers to one individual. The data is also missing when trying to connect antlers to one individual deer across time. Therefore, genetic protocols that can match sets of antlers to an individual deer are necessary.

A recent study investigated connective tissue around the pedicle (skin ring) of the cast antler (**Figure 1**) and found that connective tissue could be a reliable non-invasive way to extract DNA from South Andean Deer, *Hippocamelus bisulcus* (Venegas et al. 2020) while maintaining the integrity of the cast antler. The presence of a skin ring is critical to antler casting studies as it identifies the antler was cast within a given timeframe, and therefore can be assigned a growing year (Peterson et al. 2021). For these reasons, that connective tissue showed promise for other species like white-tailed deer but could not be used until genetic protocols are first developed and tested. The objectives of this study were to 1) establish a genetic protocol to harvest DNA from the connective tissue on cast white-tailed deer antlers and 2) determine individuality of cast antlers including in subsequent years. These protocols, to our knowledge, are the first to confirm individuality of free ranging white-tailed deer utilizing the skin ring from freshly cast antlers.

Materials and Methods

To test that the PCR primers gave the desired products, we first conducted DNA extractions on muscle tissue from female and male samples. An ~40 mg piece of muscle was macerated in digestion buffer (100mM NaCl; 10mM TrisCl, pH 8; 25 mM EDTA; 0.5% SDS; 0.1 mg/mL proteinase K) and placed in a 50ºC water bath overnight. The samples were centrifuged at 1700 rpm for 10 minutes to pellet debris and the supernatant retained. The DNA was extracted from the supernatant using an equal volume of chloroform, shaking vigorously, and centrifuging at 1700 rpm for 10 minutes. The upper aqueous phase was collected, and the DNA precipitated using 1 volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol and placing at -20ºC overnight. The samples were centrifuged at 13,000 rpm for 15 minutes, the supernatant removed, and the pellet dried. The resulting DNA pellet was resuspended in 1X TE buffer. After extraction, the DNA purity and concentration was determined using a NanoDropONE spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The samples were analyzed for the presence of mtDNA utilizing primers designed to the *d-loop hypervariable region* (forward: 5'-TCTCCCTAAGACTCAAGGAAG -3; reverse: 5'- GTCATTAGTCCATCGAGATGTC-3') (Lang 2010). To distinguish male from female, the presence of the sex determining region of the Y chromosome (*SRY*) gene was analyzed (forward: 5'-TGAACGAAGACGAAAGGTGGCTCT-3'; reverse: 5'-TACCCTATTGTGGCCCAGGTTTGT-3') (Lindsay and Belant 2008). The samples were amplified using the appropriate primers and with the Tough mix II mastermix (Thermo Fisher Scientific, Waltham, MA). The amplification conditions for mtDNA *d-loop* detection were 94ºC for 3 minutes, (94ºC for 30 sec, 60ºC for 30 sec, 68ºC for 30 sec)$_{25\ cycles}$, 68ºC for 3 minutes and hold at 4ºC. A positive reaction yielded a product at approximately 699 bp. The amplification conditions for *SRY* detection were 94ºC for 3 minutes (95ºC for 30 sec, 55ºC for 30 sec, 72ºC for 30 sec)$_{35\ cycles}$, 72ºC for 5 minutes and hold at 4ºC. A positive reaction yielded a product at approximately 350 bp. As expected, both male and female samples had mtDNA *d-loop* products at ~699 bp, but only the male sample had an *SRY* product at ~350 bp. These samples served as positive and negative controls for the antler skin ring analyses.

To determine whether the skin ring of the antler could be used for the PCR analyses and obtain results comparable to the muscle control samples, we tested 6 random antlers. The connective tissue was removed from the antlers via scraping with a with a sterile paper clip onto a clean sheet of paper for each antler. The collected sample was placed in a 1.5 mL microcentrifuge tube and stored at -80ºC. The weight of the sample was approximately 25 mg to maximize the DNA extraction. To extract DNA from the samples, the DNeasy plant and tissue extraction kit was utilized following manufacturer's instruction (Qiagen inc., Valencia, CA). Each sample was utilized for both mtDNA *d-loop* and *SRY* PCR analysis. The samples were unremarkable, in that, they all had one product at ~ 699 bp for the mtDNA *d-loop* and one product at ~350 bp for

Figure 1. Skin ring (Connective tissue) denoted by the blue arrow used as the DNA source.

SRY. These antler samples were used as the positive controls for analysis of the experimental antlers.

For the "proof of concept" that antlers from an individual deer across two years could be identified using this method, we used four antlers of interest that were believed to be the same deer collected in consecutive years. The antlers were identified as 2010 left and right (2010L/R) and 2011 left and right (2011L/R) (**Figure 2**). From the four antlers of interest believed to be from the same deer, DNA was targeted from the skin ring (Figure 1). Cast antlers 2010L/R and 2011L/R were freshly collected during their respective antler casting season and were no more than 1.5 km from each other. The DNA was extracted from the skin ring of the antlers and PCR analyses for the mtDNA *d-loop* and *SRY* genes were all done as detailed in the previous sections. Gel extraction of PCR products was necessary for sequencing and was completed using the GeneJET Extraction

Figure 2. Naturally cast antlers tested. Top antlers (left and right sides) were an assumed match set from 2010 while the bottom antlers (left and right sides) were an assumed match set from 2011. Both sets, based on antler similarities and collection proximity (<1.5 km), were assumed to be from the same deer during subsequent years. The antlers were collected from the southeastern portion of Dawson County, Nebraska, USA.

and DNA Cleanup Microkit per manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). For each sample, 20 ng of the purified PCR product along with 25 pM of forward primer for the target gene were sent to the University of Nebraska Medical Center (UNMC) Genomics Core Facility, for traditional Sanger sequencing via Genewiz. The resulting sequence files were uploaded into the NCBI Nucleotide BLAST Program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine identity of the sequences. The resulting sequence files were uploaded into the NCBI Nucleotide BLAST Program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine identity of the sequences. Alignment and sequence similarity analyses were done utilizing the EMBL-EBI Clustal Omega Multiple Sequence Alignment Tool (https://ebi.ac.uk/Tools/msa/clustalo/).

Results

The DNA was successfully extracted from the skin rings from fresh, naturally cast antlers collected in 2010 and 2011 (Figure 2). DNA yields for the samples were 10-50 ng/ul and the purity was approximately 1.8 for the 260/280 ratio for each sample. PCR amplification for *SRY* was positive in male samples (**Figure 3**, lanes 3, 5, and 6) with a product present at approximately 350 bp. Verification of mtDNA *d-loop* amplification was verified by a purified product at approximately 699 pb (Figure 3, lanes 10-12). In a number of samples, two products approximately 699 bp (Figure 3, lane 9) were detected and hypothesized to be indicative of heteroplasmy.

Doublet mtDNA *d-loop* PCR products in the 2010L/R and 2011L/R were subjected to gel purification to obtain

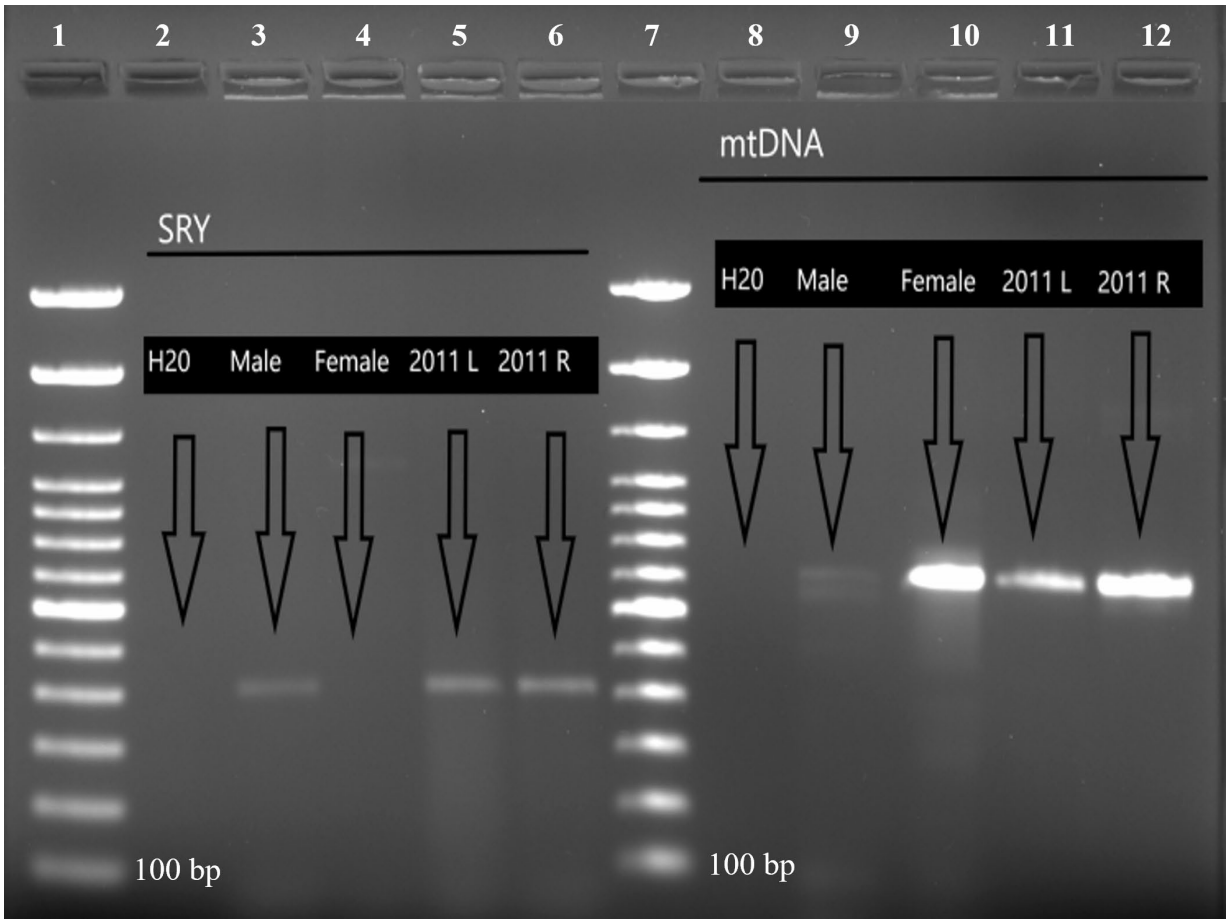

Figure 3. Confirmation of *SRY* and mtDNA *d-loop* presence via PCR. Lanes 1 & 7: 100 bp ladder; lanes 2 & 8: negative water control; lanes 3 & 9: positive control (muscle tissue from male deer); lanes 4 & 10: negative control (female muscle tissue), lanes 5 & 11: skin ring DNA from 2011L antler, lanes 6 & 12: skin ring DNA from 2011R antler. Samples in lanes 2, 5, and 6 were confirmed to be male by the presence of a PCR product at approximately 350 bp. The sample in lane 9 demonstrates two PCR products for the mtDNA *d-loop* indicating possible heteroplasmy. Samples in lanes 10-12 demonstrate the presence of the mtDNA *d-loop* as evidenced by an approximate 699 bp product. Samples in lanes 11 and 12 were the result of gel purification of the predominant ~699 PCR product from the doublet that appeared after PCR.

the predominant ~699 bp product. The purified products were sent to be sequenced and ~620 bp of useable sequence was obtained for each sample. The sequences were uploaded into the NCBI Nucleotide BLAST Program. All samples showed 99% or greater sequence identity to white-tailed deer mtDNA *d-loop* sequence (accession #AF016978.1). To determine sequence similarity between the samples, the sequences were aligned utilizing the EMBL-EBI Clustal Omega Multiple Sequence Alignment Tool (**Table 1**). Based on the antler metrics, 2010 was likely a 2.5-year-old and 2011 was likely a 3.5-year-old based on main beam lengths and mass measurements (Schoenebeck et al. 2013). Sequence similarity between antlers 2010L/R was 98.2% and 2011L/R was 97.8%. When comparing 2010L/R to 2011L/R the similarity was 96% (Table 1).

Table 1. Percent sequence similarity as determined by the EMBL-EBI Clustal Omega Multiple Sequence Alignment Tool. The percentages in bold are for the match sets for 2010 and 2011.

	2010 Left	2010 Right	2011 Left	2011 Right
2010 Left	100			
2010 Right	**98.2**	100		
2011 Left	94.9	96	100	
2011 Right	95.1	94.4	**97.8**	100

Discussion

The methods used in this study demonstrate that pure (260/280 ratio of 1.8) and a tangible concentration of DNA is achievable using the skin ring. DNA quantification and qualification are necessary to avoid low confidence results, high costs, and wasting samples. Before using DNA in any genetic analysis, the quality and usability must be determined through quality indicators that assess DNA purity and integrity. The 260/280 nm ratio of absorbance is used to assess purity. A ratio of 1.8 is generally accepted as "pure" for DNA. A lower ratio indicates contamination from proteins or due to traditional DNA extraction techniques using phenol. Higher than 1.8 typically indicates RNA contamination (Glasel 1995, Lucena-Aguilar et al. 2016). None of these were an issue in this study, as the commercial kit used for DNA extraction minimizes contamination from these sources and all our samples measured a 260/280 nm ratio of 1.8, indicating pure DNA.

Another concern when performing these types of studies is degradation of DNA prior to downstream applications such as PCR analysis. This study utilized gentle scraping of the skin ring, which is a non-invasive technique compared to the conventionally used drilling technique. In other studies that aimed at extracting DNA from antlers, a drill bit was used to grind out the antler to obtain sample to use for extraction. This technique was done for weathered antlers from white-tailed deer (O'Connell and Denome 1999, Lopez and Beier 2012), native red deer populations (*Cervus elaphus italicus*) from Italy (Greco et al. 2022) and Scotland (Huisman et al. 2016), seventeen different deer species that were up to 40 years old and many listed on the IUCN Red list (Hoffmann et al. 2014), and 200-year-old red deer antlers (Hoffmann and Griebeler 2013), to name a few. Unfortunately, this technique is highly problematic for downstream genetic applications. Drilling into bone without cooling leads to immediate increases of temperature over 200°C (Brand et al. 2013). This is a major issue, as complete DNA degradation occurs above 190°C (Karni et al. 2013). Using degraded DNA as a template in PCR leads to products larger than expected due to amplification of overlapping fragments, illegitimate amplification of non-targets, decreased production of target amplicon, and limitations in the incorporation of dNTPs that leads to a reduction in the efficiency of PCR (Golenberg et al. 1996). For these reasons, DNA in this study was experimentally extracted from the soft connective tissue (skin ring present on freshly cast white-tailed deer antlers). This yielded high quality DNA that allowed successful PCR amplification of both *SRY* and mtDNA *d*-loop regions. The use of the skin ring as a source of DNA has also been utilized from antlers of South Andean Deer (Venegas et al. 2020), but to our knowledge this is the first time it has been performed in white-tailed deer. Antler connective tissue could likely be explored as a viable means to extract DNA from other deer species, museum specimens, and freshly shed antlers. PCR amplification of the *SRY* gene was successful, as indicated by the ~350 bp product found in the male samples (Figure 3). This allowed us to determine that our DNA extraction from the skin ring yielded usable DNA and confirmed the PCR protocols we were using were consistent with other published research (Lindsay and Belant 2008).

We proceeded to amplify the mtDNA *d-loop* via PCR using published primers and protocol for white-tailed deer (Lang 2010). With this protocol, PCR amplification of mtDNA *d*-loop demonstrated PCR doublets (Figure 3, lane 9), which we hypothesize may be heteroplasmy. This may account for the 2% difference between the left and right antlers from the same year and the 4% difference between 2010 and 2011. This finding is novel, in that, there are no other published accounts of determining mtDNA *d-loop* sequence similarity between matched sets of deer antlers to determine individuality. There is also no published level of percentage of sequence similarity that is accepted to state that the mtDNA is from the same individual. A single heteroplasmic individual can be heteroplasmic or homoplasmic for different tissues (Parakatselaki and Ladoukakis 2021), which makes determining the level of sequence similarity of a single mitochondrial gene sequence in a suspected heteroplasmic individual difficult. Fortunately, in populations, heteroplasmy is known to be rare. This was shown in a study of the *d-loop* control region in white-tailed deer populations from 30 localities in the US and Guatemala. In this study, the data showed that only 7.8% of the total sample population (n = 1,135) tested, demonstrated heteroplasmy in this region (Purdue et al. 2006). The results presented also show that the heteroplasmy was only present in the male experimental samples, while being absent in the unrelated female and male muscle tissue, as well as the random unrelated antlers that were used as control samples in this study. When considering the increase in sequence difference from 2010 to 2011, this is consistent with data from humans demonstrating that mtDNA heteroplasmies increase with age (Zhang et al. 2017). When considering heteroplasmic individuals can vary in level of sequence similarity between tissues, the rarity of heteroplasmy in populations, that heteroplasmy was only suspected in our

experimental samples, that heteroplasmic mutations increase with age, and the other phenotypic metrics used, the hypothesis that the experimental antlers came from the same individual deer appears valid.

The hypothesized heteroplasmy seen in this study may be due to the high mitochondrial count per cell. Most somatic cells contain hundreds to thousands of mtDNA molecules, creating a heteroplasmic condition in which two genotypes exist within an individual. When researching mtDNA, it was originally feared that heteroplasmy may be extensive and complicate mtDNA research (Avise et al. 1987). At this time, more samples will need to be analyzed to determine how extensive heteroplasmy is in the white-tailed deer population of Nebraska. A recent study from China found that antler growth in cervids may be a form of self-controlled cancer growth (Wang et al. 2019). Antlers grow at a rapid rate and may be the fastest regenerative tissue and are similar to cancer growth tumors in terms of speed because of elevated proto-oncogenes. Heteroplasmy is also common within cancer-based diseases, this could be a possible contributing factor of heteroplasmy in antlers due to replication mistakes (Wang et al. 2019). In future research, the hypothesized heteroplasmy needs to be validated. To do this, the minor band of the doublet needs to be sequenced to verify heteroplasmy. This could also be done by sequencing more antlers from Nebraska and comparing them to antlers from other regions or states by doing a molecular phylogeny as was done in sika deer (*Cervus nippon*) in Japan (Nagata et al. 1999). In addition to allowing us to determine if the heteroplasmy exists, it would allow us to determine if the heteroplasmy is specific to regions within Nebraska.

The establishment of these protocols offer a technique to confidently assign individuality to suspected match sets and antlers from the same white-tailed deer over multiple seasons. Determining individuality from naturally cast antlers allows researchers to maximize sample size while avoiding pseudoreplication. Advancing our techniques continue to provide wildlife biologists and game managers a less intrusive means to obtain data at the individual and herd level as they set future management goals and harvest regulations.

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