A PCR-based assay for discriminating *Cervus* and *Rangifer* (Cervidae) antlers with mitochondrial DNA polymorphisms

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**ABSTRACT:** This study describes a method for discriminating *Rangifer* antlers from true *Cervus* antlers using agarose gel electrophoresis, capillary electrophoresis, quantitative real-time PCR, and allelic discrimination. Specific primers labeled with fluorescent tags were designed to amplify fragments from the mitochondrial D-loop genes for various *Cervus* subspecies and *Rangifer tarandus* differentially. A 466-bp fragment that was observed for both *Cervus* and *Rangifer* antlers served as a positive control, while a 270-bp fragment was specifically amplified only from *Rangifer* antlers. Allelic discrimination was used to differentiate between *Cervus* and *Rangifer* antlers, based on the amplification of specific alleles for both types of antlers. These PCR-based assays can be used for forensic and quantitative analyses of *Cervus* and *Rangifer* antlers in a single step, without having to obtain any sequence information. In addition, multiple PCR-based assays are more accurate and reproducible than a single assay for species-specific analysis and are especially useful in this study for the identification of original *Cervus* deer products from fraudulent *Rangifer* antlers.

**Key words:** agarose gel electrophoresis, capillary electrophoresis, *Cervus* antlers, *Rangifer* antlers, real-time PCR,


**INTRODUCTION**

*Cervi Parvum Cornu* (*Cervus* antlers) is a well known Korean traditional medicine derived from the young antlers of male *Cervus* deer, consisting of horizontal slices of the velvet-covered antler. Specifically, it has been reported to have anti-aging, androgenic, gonadotrophic, PG, hematopoietic, and immunomodulatory activities on humans (Suh et al., 1999; Kim et al., 2004). *Rangifer* (reindeer and caribou, subfamily Odocoileinae) antlers, on the other hand, are rarely consumed as a food, or used as a traditional medicine (KFDA, 2011).

The efficacy and price of *Cervus* antlers varies according to the grade of the antler slices, as well as the sources of the slices. However, since it is difficult to differentiate the antlers of *Rangifer tarandus* (reindeer) from *Cervus* antlers, and since the morphological differences between the sliced antlers are insignificant, slices from the former are often mixed with, or sold as, *Cervus* antlers. The application of PCR techniques, which tend to be more specific, sensitive, and applicable even to heat processed products, has been extensively investigated (Bottero et al., 2003; Dalmasso et al., 2004). Molecular methods, such as PCR-based assays, have also been widely used to accurately distinguish between meats from different species (Kesman et al., 2009).

The mitochondrial DNA (*mtDNA*) sequences of wapiti (*C. elaphus nelsoni*), red deer (*C. elaphus*), and sika deer (*C. nippon*) of *Cervus*, and caribou of *Rangifer* have previously been compared (Polziehn and Strobeck, 1998). Moreover, variation in *mtDNA* has been investigated in wildlife forensic science, for species identification from tissues (Cronin et al., 1991) of reindeer and caribou from Alaska (Cronin et al.,

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Kim et al. 1995). Similarly, mtDNA and microsatellite DNA variation have been investigated to establish the relationship between domestic reindeer and wild caribou (Cronin et al., 2006). Gilbert et al. (2006) used mitochondrial and nuclear DNA markers to elucidate the biogeography and evolution of Cervidae for taxonomic and phylogenetic analysis. Therefore, the aim of this study was to develop a method to rapidly and specifically discriminate Rangifer antlers from true Cervus antlers by using both agarose gel and capillary electrophoresis, real-time PCR, and allelic discrimination methods.

**MATERIALS AND METHODS**

All experimental protocols were approved by the Korean Institute of Oriental Medicine Institutional Animal Care and Use Committee.

**Samples and DNA Preparation**

Out of 50 antlers imported to Korea from Russia, Canada, China, New Zealand, and Alaska, 15 samples that had been identified as Cervus or Rangifer antlers were used in this study. These included the following: 12 different subspecies of Cervus (i.e., *C. elaphus sibericus*, 2 samples; *C. elaphus nelson*, 2 samples; *C. elaphus canadensis*, 1 sample; *C. elaphus manitobensis*, 1 sample; *C. elaphus*, 2 samples; *C. elaphus bactrianus*, 1 sample; *C. elaphus lepisanus*, 2 samples; and *C. elaphus manchuricus*, 1 sample).

*Figure 1.* Nucleotide sequences of the mitochondrial DNA (mtDNA) control region used to distinguish Cervus and Rangifer antlers. Primer-probe sets are marked by square boxes; the sequences can be found in Table 1. See online version of figure for color.
Discriminating *Cervus* and *Rangifer* antlers

2 samples, *C. nippon*; 2 samples), and 3 samples of *R. tarandus* from the subspecies *R. tarandus tarandus* (Figure 1). The species were confirmed by comparing the nucleotide sequences of the D-loop region of mtDNA with known nucleotide sequences in the NCBI GenBank database (*C. elaphus sibericus*: AF058371; *C. elaphus nelson*: AF016965, AF016962; *C. elaphus canadensis*: AY970666; *C. elaphus mantiobensis*: AF016958; *C. elaphus bactrianus*: AF296822; *C. nippon*: AF016974; *R. tarandus tarandus*: AF096449).

Genomic DNA was extracted from 100 mg of antler slices using a QIAamp DNA plant kit (Qiagen GmbH, Hilden, Germany). The DNA purity testing and quantitative analyses were conducted using an ND-1000 spectrophotometer (NanoDrop Tech., Wilmington, DE).

**PCR and DNA Sequencing**

To amplify the D-loop region of mtDNA from the extracted DNA, CST2 (5'-TAATATACCTGGTCTTGAAACC-3') and CST39 (5'-GGGTCGAAAGGCTG GGACCAAACC-3') primers were used (Polziehn and Strobeck, 1998). For PCR amplification, 20 ng/μL template DNA and 1 μL each of 10 pmol CST2 and CST39 primers were mixed with 2× PCR Pre-Mix (Solgent, Daejeon, Korea) in a total reaction volume of 25 μL. The PCR conditions were as fol-
Figure 2. Sequence pair distance for the 15 samples of Cervus and Rangifer antlers, based on D-loop sequence data, according to the ClustalW method (Thompson et al., 1994).

Figure 3. Phylogenetic tree for the 12 Cervus and 3 Rangifer antlers, based on D-loop sequence data, according to the ClustalW method (Thompson et al., 1994).

Table 1. Oligonucleotide primers and probe sequences for electrophoresis-based PCR and real-time PCR

<table>
<thead>
<tr>
<th>Method</th>
<th>Primer / Probe</th>
<th>Oligonucleotide sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose gel electrophoresis</td>
<td>CST2</td>
<td>TAA TAT ACT GGT CTT GTA AAC C</td>
</tr>
<tr>
<td></td>
<td>JER1</td>
<td>CAT ATA TGT ACG TTT ATA AAA TAT</td>
</tr>
<tr>
<td></td>
<td>L14724-tm58²</td>
<td>TTG ATA TGA AAA ACC ATC GTT G</td>
</tr>
<tr>
<td></td>
<td>H15149-tm58²</td>
<td>TCA GAA ATG ATA TTT GTC CTC A</td>
</tr>
<tr>
<td>Capillary electrophoresis</td>
<td>CST2</td>
<td>TAA TAT ACT GGT CTT GTA AAC C</td>
</tr>
<tr>
<td></td>
<td>C-JER1</td>
<td>JOE-CAT ATA TGT ACG TTT ATA AAA TAT</td>
</tr>
<tr>
<td></td>
<td>L14724-tm58³</td>
<td>TTG ATA TGA AAA ACC ATC GTT G</td>
</tr>
<tr>
<td></td>
<td>C-H15149-tm58³</td>
<td>FAM-TCA GAA ATG ATA TTT GTC CTC A</td>
</tr>
<tr>
<td>Quantitative assay</td>
<td>QF</td>
<td>ATA TTA TGT ATA GTA CAT TAA ATT ATA TGC CCC ATG CTT</td>
</tr>
<tr>
<td></td>
<td>QR</td>
<td>CGC ATG TTG ACA AGA AAG GAT TTG A</td>
</tr>
<tr>
<td></td>
<td>QFAM</td>
<td>6FAM-CCA TGT ACG ATC AAT ATT-MGBNFQ</td>
</tr>
<tr>
<td>Allele discrimination</td>
<td>AF</td>
<td>ACA TAA CTG TGG TGG CAT ACA TTT GGT</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>AAG ATG CAG TTA AGT CCA GCT ACA A</td>
</tr>
<tr>
<td></td>
<td>AFAM</td>
<td>6FAM-ACG GCC ATA GCT GAG-MGBNFQ</td>
</tr>
<tr>
<td></td>
<td>AVIC</td>
<td>VIC-ACG GYC ATT GCT GAG-MGBNFQ</td>
</tr>
</tbody>
</table>

1QF, Quantitative assay-Forward primer; QR, Quantitative assay-Reverse primer; QFAM, Quantitative assay-a FAM-labeled probe; AF, Allele discrimination-Forward primer; AR, Allele discrimination-Reverse primer; AFAM, Allele discrimination-a FAM-labeled reporter TaqMan MGB probe; AVIC, Allele discrimination-a VIC-labeled reporter TaqMan MGB probe

2Primer sequences according to the original method (Polziehn and Strobeck, 1998)

3Primer sequences according to the original method (Masuda et al., 1996)
2.05 (MiraiBio, South San Francisco, CA) and BioEdit Sequence Alignment Editor V. 7.0.9.0 (Ibis Biosciences, Abbott Park, IL). Sequence distance (Figure 2) and phylogenetic tree analyses (Figure 3) were performed using MegAlign V. 7.2.0 (DNASTAR, Inc., Madison, WI).

Electrophoresis-Based PCR Assay

Agarose Gel Electrophoresis. For analysis by agarose gel electrophoresis, primer sets CST2/JER1 and L14724-tm58/H15149-tm58 (Table 1) were used to amplify sequences in the mitochondrial D-loop and cytochrome b regions, respectively; the latter primer set had been modified from Masuda et al. (1996). The PCR reaction mixtures contained 10× reaction buffer (Applied Biosystems), 1.5 mM MgCl$_2$, 0.2 mM dNTPs, and 1.25 U AmpliTaq Gold polymerase (Applied Biosystems). The PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 7 min.

The PCR products were electrophoresed on a 2% agarose gel (Amresco, Cleveland, OH) with 0.5 μg/mL ethidium bromide in 0.5× TBE buffer (45 mM Tris-borate and 1 mM EDTA; pH 8.0). Gels were stained with ethidium bromide and visualized under ultraviolet light.

Capillary Electrophoresis. For analysis by capillary electrophoresis, the C-JER1 primer was 5′-end-labeled with a fluorescent dye, 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxy-fluorescein (JOE), while the H15149-tm58 primer was labeled at the 5′-end-labeled with 6-carboxyfluorescein (6-FAM; Table 1).

For PCR amplification, 10 pmol of each primer (CST2, C-JER1, L14724-tm58, and C-H15149-tm58) and 1 to 10 ng of template DNA were added to a 2× PCR Premix (Solgent) in a total reaction volume of 30 μL. The conditions for PCR, performed on a PTC-200 thermal cycler (MJ Research, Waltham, MA), were as follows: initial denaturation at 95°C for 15 min; 35 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 30 s, and extension at 72°C for 40 s; followed by a final extension step at 72°C for 5 min.

Hi-Di Formamide (Applied Biosystems; 14.7 μL) and 0.3 μL of GeneScan-500ROX (Applied Biosystems) were added to the PCR products. Samples were analyzed on a Genetic Analyzer 310 (Applied Biosystems); electrophoresis was conducted at 15 kV for 24 min using Filterset F and POP-4 matrix.

Quantitative Assay and Allelic Discrimination by Real-Time PCR

Quantitative Real-Time PCR. Primers QF and QR, which allow specific amplification of Rangifer DNA, and a FAM-labeled probe (QFAM) were used for quantitative PCR analysis of mtDNA from Rangifer antlers (Table 1), using an ABI 7500 Realtime PCR system (Applied Biosystems) and Sequence Detection Software (Version 2.0). The PCR reaction contained 12.5 μL of Taqman Universal PCR Master Mix, 1.25 μL of 20× Taqman primer/probe reagent, 8.25 μL of sterilized distilled water, and 120 ng DNA in a 96-well optical reaction plate. The reaction was incubated at 50°C for 2 min, which was followed by 40 cycles of activation at 95°C for 10 min, denaturation at 95°C for 15 s, and incubation at 60°C for 1 min.

Absolute Quantification in the SDS software package was used to analyze the data from the quantitative assay; the standards for the assay were established by subcloning 1142 bp of the D-loop region of Rangifer antlers in the pGEM-T Easy Vector (Promega). The copy number was calculated according to the formula of Tobe and Linacre (2008).

Allelic Discrimination. For distinction of mtDNA PCR products from Cervus and Rangifer antlers by allelic discrimination, forward (AF) and reverse (AR) primers were used in conjunction with allele-specific probes, viz., probe 1 (AFAM), a FAM-labeled reporter TaqMan MGB probe that recognizes allele 2 with thymine at position 742 in Rangifer antlers, and probe 2 (AVIC), and a VIC-labeled reporter TaqMan MGB probe that recognizes allele 1, without the thymine. These reagents were prepared as a 40× primer/probe mix using the Assays-by-Design Service from Applied Biosystems. The sequences of the primers and probes are shown in Table 1.

For PCR, 5 μL TaqMan Genotyping Master Mix (Applied Biosystems), 0.25 μL 40× primer/probe mix (Applied Biosystems), 3.75 μL sterilized distilled water, and 5 ng DNA were mixed. Real-time PCR amplification was performed with an ABI 7500 Real-time PCR system from Applied Biosystems. The pre-PCR step of 30 s at 60°C was followed by 40 cycles of incubation at 95°C for 10 min, denaturation at 92°C for 15 s, and incubation at 60°C for 90 s, followed by a post-PCR step at 60°C for 30 s.

The Sequence Detection Software 7500 (Ver. 2.0) software package was used to analyze the data from the allelic discrimination assay. Immediately after a run, the software automatically analyzed the data using the default analysis setting and then displayed the Allelic Discrimination Plot on screen.
RESULTS

Comparisons of Mitochondrial D-Loop Sequence of Cervus and Rangifer Antlers

To confirm that we had amplified the D-loop sequence of the 12 Cervus and 3 Rangifer samples, homology between the sequences of these amplified fragments and known nucleotide sequences in the NCBI GenBank database was compared. All samples showed 99% identities compared with the GenBank sequence, except for the C. nippon sample, which showed 95% similarity. The D-loop sequences of 3 R. tarandus samples were found to be identical. Moreover, the sequence of the R. tarandus samples analyzed here differed from that of R. tarandus tarandus (AF096449) at 14 positions, and was, therefore, deposited in GenBank as a new R. tarandus D-loop sequence (accession number: AY970667). Figure 1 shows the nucleotide sequences of the D-loop of the 15 samples analyzed in this study. Sequence pair distance (Figure 2) and phylogenetic tree (Figure 3) analyses were also performed for the 15 samples, according to the ClustalW method, using the MegAlign program. The observed differences in the D-loop sequences were then used to design primer sets that allow discrimination between Rangifer antlers and true Cervus antlers.

Discrimination of Rangifer Antlers from Cervus Antlers Using Electrophoresis-Based PCR Assay

The PCR products derived from the antlers were separated by agarose gel electrophoresis and identified by size: a 466-bp fragment was used as an internal control to verify success of amplification in samples from the 12 Cervus, as well as from the 3 Rangifer antlers, and derived from amplification of cytochrome b. A 270-bp fragment was specific for the 3 Rangifer antlers (Figure 4); this fragment derived from specific amplification of the mtDNA D-loop.

The accuracy of the size of the amplified PCR products size was confirmed by capillary electrophoresis. As with agarose gel electrophoresis, a 270-bp light green fluorescent fragment was detected only from the 3 Rangifer antlers, whereas both 12 Cervus and 3 Rangifer samples produced a 466-bp blue fluorescent product (Figure 5).

Figure 4. Discrimination of individual Cervus antlers (lanes 1 to 12) and Rangifer antlers (lanes 13 to 15) by agarose gel electrophoresis of PCR products.

Figure 5. Electropherogram of a multiplex PCR for distinguishing between Rangifer antlers (A) and Cervus antlers (B) by capillary electrophoresis. The X-axis shows the size in base pairs. The Y-axis shows the intensity of fluorescence in relative fluorescent units. The upper panel (A) shows the product with a 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxy-fluorescein (JOE) fluorescent tag. The lower panel (B) shows the product with a fluorescein (FAM) fluorescent tag. See online version of figure for color.
Quantitative Assay and Allele Discrimination for Rangifer Antlers by Real-Time PCR

For real time-PCR analysis, the threshold \( (C_T) \) value ranged from 13 to 21 when the copy number was \( 1.4 \times 10^{10} \) to \( 1.12 \times 10^8 \) (data not shown). To differentiate between Rangifer and Cervus antlers, a primer and probe set specific to Rangifer antlers were used to amplify mtDNA and to quantify mtDNA copy number. While no amplification was evident in the Cervus samples, the copy numbers for the Rangifer samples ranged between \( 2.6 \times 10^9 \) and \( 8.1 \times 10^8 \), corresponding to \( C_T \) values of 16.4261 and 18.3038, respectively (data not shown). Greater template concentrations, and thus greater copy numbers, were associated with lower \( C_T \) values. The quantitative values from this method can be applied for quick and precise real-time forensic analysis, even when DNA content is limited and of low quality.

Allelic discrimination detects variant forms of the same gene that differ by nucleotide substitution, insertion, or deletion. Each allele can be distinguished by a PCR assay using different fluorescent reporter dyes attached to the oligonucleotides in the reaction. To differentiate the antlers, we used FAM or VIC fluorescent dyes on the forward and reverse primers; these allowed the detection of alleles 1 and 2 in the Cervus and Rangifer samples, respectively (Figure 6).

![Allelic Discrimination Plot](image)

**Figure 6.** Allelic discrimination plot for the 15 samples of Cervus and Rangifer antlers. (A) Rangifer antlers; (B) Cervus antlers; (C) control sample, with no DNA template. The X-axis shows the samples that were detected with VIC fluorescence as homozygous for allele 1. The Y-axis shows the samples that were detected with FAM fluorescence as homozygous for allele 2. See online version of figure for color.

DISCUSSION

In this study, we developed a method to discriminate rapidly and accurately between Cervus and Rangifer antlers, exploiting differences in the nucleotide sequences of the mtDNA. Various subspecies and haplotypes of Cervus and Rangifer antlers have been studied in the past (Kuwayama and Ozawa, 2000; Mahmut et al., 2001; Cronin et al., 2009; Puputti and Niskanen, 2009). These studies have primarily used mtDNA sequences for the phylogenetic analysis of these groups. In particular, as mtDNA shows an adequate degree of intra- and interspecies variability and high copy number in each cell (Kesmen et al., 2009), it is useful for the classification and analysis of distribution of Cervus species.

A study of the taxonomic diversity of 40 deer species using mitochondrial and nuclear DNA has been reported for the Cervidae family (Gilbert et al., 2006). In addition, studies of the phylogenetic position of giant deer (Lister et al., 2005) and of the molecular phylogeography of red deer (Mahmut et al., 2001) also have dealt primarily with the phylogenetic evolution of the Cervidae family. However, there have been virtually no studies on developing a method to differentiate between Cervus and Rangifer antlers. Biological samples from unknown species are encountered frequently in forensic science investigations (Tobe and Linacre, 2008). In particular, a method is needed to analyze samples that have been cut up and mixed, or contaminated, without having to resort to sequence analysis. Moreover, given that Cervus antlers are used both as food and in traditional medicine, but are difficult to differentiate from Rangifer antlers morphologically, consumers may mistakenly consume the wrong antlers. Therefore, there is a demand for a molecular genetic method that can reliably differentiate between antlers from these 2 species. Forensic DNA analysis has previously been applied to deer (Jones et al., 2000; Poetsch et al., 2001).

Rangifer antlers derive from either mountain reindeer (wild and semi-domesticated reindeer; Rangifer tarandus tarandus), or forest reindeer (R. tarandus fennicus), with postcranial measurements typically used to differentiate between these species (Puputti and Niskanen, 2009). This study did not find intraspecific differences in reindeer but used a PCR-based assay in a forensic capacity to distinguish between sliced Cervus and Rangifer antlers. Previously, to investigate the genetic variation in Odocoileus hemionus (mule deer), forensic DNA had been performed using a multiplex PCR reaction with fluorescently labeled primers (Jobin et al., 2008). In our study, specific regions of mtDNA from Cervus and Rangifer antlers were similarly detected, using capillary electrophoresis. Since DNA sequencers detect the fluorescence from fluorescently labeled primers in amplified...
sequences (Liljander et al., 2009), the size of nucleo-
tides fragments can be determined more accurately than
by gel electrophoresis. In addition, by using multiplex
PCR with different fluorescent labels, several amplified
products can be effectively analyzed simultaneously. As
a result of these advantages, capillary electrophoresis is
widely used in the fields of medicine, forensic medicine,
pharmacology, and food analysis (Kim et al., 2005; King
et al., 2005). For forensic identification of humans, Liu
et al. (2008) demonstrated greater amplification effi-
ciency and a shorter separation time in separating 106- to
259-bp DNA fragments with a 9-plex capillary electro-
phoresis system. For this study, a 466-bp product that
identifies both Cervus and Rangifer antlers and a 270-bp
product that specifically identifies Rangifer antlers were
analyzed together; this allowed the 2 antlers types to be
distinguished in a single step.

The ability to calculate copy number of mtDNA in fo-
rensic and ancient DNA samples by quantitative assay has
been improved significantly with fluorogenic probes and
quantitative real-time-PCR detection systems (Alonso et
al., 2004). The rapid and quantitative assaying of mtDNA
copy number is used in many fields, including mol-
ecular, evolutionary, and forensic biology (Von Wurmb-
Schwark et al., 2002). Although species cannot easily be
distinguished from Cervus antlers, we effectively used
real-time PCR and capillary electrophoresis to distin-
guish between Cervus and Rangifer antlers by genotype;
we simultaneously determined the precision and reliabil-
ity of real-time PCR to quantify mitochondrial genome
copy number. In addition to genotyping on the basis of
differences in the nucleotide sequence of mtDNA, real-
time PCR can be used to rapidly analyze material before
sequence analysis, especially when the DNA is highly de-
graded or the amount limited. The use of real-time PCR
for quantitative assays and allele discrimination has short-
ened the analysis time by eliminating post-PCR process-
ing; the risks of nucleotide sequence errors and genomic
DNA contamination is also reduced in this way, making it
is appropriate for genotyping of mtDNA.

Single nucleotide polymorphism genotyping can be
used as an end point assay to determine the genotype of an
unknown sample. In our study, it was used to discriminate
between Cervus and Rangifer antlers by distinguishing
them according to the alleles present. This multiple PCR-
based analysis may be a powerful tool for facilitating iden-
tification of specific species, since the use of several assays
generates more reproducible results than a single assay.

In conclusion, this study developed a fast and accu-
rates method using several PCR-based assays, to differen-
tiate reindeer antlers from those of other subfamilies of
der. This method can be used for quality control of deer
products by discriminating effectively and quickly be-
tween Cervus and Rangifer antlers, even as antler slices.

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