

Full Length Research Paper

Multiplex polymerase chain reaction (PCR) and fluorescence-based capillary electrophoresis for identification of deer species from antlers

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Multiplex polymerase chain reaction (PCR) and fluorescence-based capillary electrophoresis (CE) of blood and tissue samples have been used to distinguish between deer species such as red deer, sika deer, wapiti and reindeer. We constructed 4 species-specific primers by using the D-loop of mitochondrial DNA and cytochrome *b* as an internal PCR control. The amplified species-specific product lengths of 199, 299, 245 and 375 bp for red deer, sika deer, wapiti subspecies and reindeer, respectively were detected from mitochondrial D-loop DNA fragments. The specificity was confirmed by analysis of blood and tissue samples of 4 deer antlers and 8 other samples of mammalian DNA (mouse, rat, pig, chicken, cat, cow, dog and human DNA). The specificity and accuracy of the multiple primers for identification were assessed at various concentrations and from mixed samples. In this study, we established a method for the identification of deer species using deer antlers.

Key words: Deer antler, identification, mitochondrial DNA, multiplex polymerase chain reaction (PCR).

INTRODUCTION

Members of the *Cervus* species (*Cervi Parvum Cornu*) are found worldwide. Morphological analyses of *Cervus* species, revealed that the European red deer (*Cervus elaphus*), the wapiti (*Cervus elaphus* subspecies in Asia and North America), and the sika deer (*Cervus nippon*) are monophyletic (Kuwayama and Ozawa, 2000). In terms of overall morphology, they are very similar to one another, except in body size and antler morphology (Geist, 1971; Zima et al., 1990; Kuwayama and Ozawa, 2000). Previous studies that compared allozymes and mtDNA between European red deer and North American wapiti revealed a clear separation between the 2 groups (Dratch and Gyllensten, 1985; Cronin, 1992; Polziehn et al., 1998). The North American elk or wapiti, were confused in name as they were similar in size to

European elk (*Aceds alces*, moose). Furthermore, the term "elk" is widely used in North America, leading to confusion at an international level. Therefore, *C. elaphus* subspecies in eastern Asia, Siberia and North America were termed "wapiti" (Polziehn et al., 1998; Pitra et al., 2004). Although, the reindeer (*Rangifer tarandus*), which belongs to the whitetail subfamily (*Odocoileinae*), is completely different from sika deer, red deer and wapiti (Geist, 1982), its cutting shape is similar to those of the *Cervus* species.

Deer products from organs or tissues of deer have been widely used as anti-aging, androgenic, gonadotrophic, prostaglandin, hematopoietic and immunomodulatory agents in human being (Suh et al., 1999; Kim et al., 2004). The quality control of deer products mostly depends upon traditional methods which mainly include macroscopical identification and microscopical examination, and physical and chemical experiment. However, the application of these methods is restricted in evaluating deer products subjected to

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different technological processes. The need for alternative analytical approaches has prompted numerous studies. The application of polymerase chain reaction (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 polymerase chain reaction (PCR)-based assays, have been used widely to accurately distinguish different meat species (Kesman et al., 2009).

Since deer products are expensive and limited, merchants usually substitute them with organs or tissues from other animals of Cervidae, such as reindeer (*R. tarandus*) and red deer (*C. elaphus*, Western lineage of *C. elaphus*) to obtain lots of economic profits. Therefore, it is very necessary to identify deer products in order to inhibit the phenomenon. Many researchers identified different animals of Cervidae by using species-specific PCR method (Fajardo et al., 2007; Liu et al., 2001; Tang et al., 2001, 2002). Species-specific PCR can selectively detect DNA sequences from a mixed sample and offer the advantages of being less expensive and more useful for routine analysis of large numbers of samples. Wu et al. (2005) and Li et al. (2006) identified sika deer and red deer by sequence analysis in the forensic identification. Most recently, researchers (Dalmasso et al., 2004; Ghovvati et al., 2009; Lin and Hwang, 2008) developed multiplex PCR to detect animal ingredients in feedstuffs or food products. This method provides a more precise detection of species-origin for complex samples, and is more labour-saving than using each pair of species-specific primers separately for the samples. Due to rapidly and accuracy of the PCR, it has been considered as a method to detect genetic differences among species and for species identification. Therefore, the aim of the present study was to develop a multiplex PCR and fluorescence-based capillary electrophoresis for rapid and accurately identification of deer species using antlers.

MATERIALS AND METHODS

Sample collection

Blood of red deer, sika deer, wapiti and reindeer were collected from Seoul Grand Park (Republic of Korea), and the antler samples were collected from different individual farms in the Republic of Korea. Imported commercial materials were obtained from Russia, China and Alaska. Blood samples from 7 animals (mouse, rat, pig, chicken, cat, cow and dog) and human beings were obtained for use as negative control.

DNA isolation and sequencing

DNA was extracted from blood or tissue using the QIAamp DNA microkit (Qiagen, Germany) according to the protocol described by the manufacturer. Three primers were used for sequencing following amplification of the D-loop region of mtDNA in red deer,

sika deer, wapiti, and reindeer. The D-loop region was amplified by PCR using the CST2 primer and CST39 primer (Polziehn et al., 1998), while the SeqR primer (5'-atg tcc tgt gac cat tga ct-3') was used to sequence the amplified D-loop region. The D-loop region was sequenced using the Perkin-Elmer DNA Terminator Cycle Sequencing Ready Reaction Kit, DNA amplifier (GeneAmp PCR System 9700, Applied Biosystems, USA), and an ABI 310 genetic analyzer (Applied Biosystems).

D-loop region sequences were obtained from the Genbank databases for wapiti, red deer, sika deer and reindeer. The sequences were compared with the test sequences to identify the animal and the individually confirmed sequences were analyzed and given the following accession numbers: AF016972, AF016973 (*C. elaphus*), AF016979 (*C. elaphus nelsoni*), AF016974, AB012371, AB012373, AB186347 (*C. nippon*), AF096441, and AF096413 (*R. tarandus groenlandicus*).

Primers designing

For the amplified products, the lengths of the D-loop sequence were: 1135, 1288, 1209, and 1142 bp for red deer, sika deer, wapiti, and reindeer, respectively. We used the different species-specific primers in a single sample to develop a "multiplex" method for simultaneous analysis. The forward primer (FOR) was designed for the common sequence. The reverse primers were designed for species-specific sequences. Accordingly, the multiplex PCR was developed by the incorporation of one tissue-specific primer for each species, namely RED for red deer, NIP for sika deer, WAP for wapiti and REIN for reindeer. The PCR primer sequences and the expected product sizes are shown in Table 1.

PCR amplification and gel electrophoresis

Reactions were performed as single amplification reactions that were combined in one multiplex reaction in a single tube. All reactions were carried out in a total final volume of 25 μ l using 1 ng of DNA as a template. The reaction mixtures contained 10 \times reaction buffer (Applied Biosystems), 1.5 mM MgCl₂ and 0.2 mM dNTP mixture along with 1.25 unit AmpliTaq Gold polymerase (Applied Biosystems). The DNA was amplified in a GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: initial denaturation at 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; with a final extension step at 72°C for 7 min.

The internal control primers were L14724 and H15149 (Masuda et al., 1996), these primers were subjected to amplification with the mitochondrial cytochrome *b* gene. The following specific primers were used in this study: RED (anti-sense; T_m value 53°C), NIP (anti-sense; T_m value 53°C), WAP (anti-sense; T_m value 53°C) and REIN (anti-sense; T_m value 54°C). Clustal -W (ver. 1.75) was used for comparative analysis of the sequences. Primer 3 (<http://www.bioneer.co.kr/tools/>) was used to produce the primers. The amplified product was electrophoresed in 2% agarose gel (Amresco, USA) containing 0.5 μ g/ml EtBr using 0.5 \times TBE buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.0), and the amplified results were confirmed using an ultraviolet (UV) transilluminator.

Capillary electrophoresis

We used a pair of specific primers in the ABI 310 Genetic Analyzer (Applied Biosystems). Each amplified sample was added to 14.7 μ l Hi-Di formamide and 0.3 μ l of GeneScan 500 (ROX) internal lane standard (Applied Biosystems). Tubes were heated at 95°C for 5 min and snap cooled on ice for at least 3 min. The samples were

Table 1. Multiplex PCR primer sequences.

Region	Primer name	Primer sequences	Observed sizes (bp)
D-loop	CST2 forward	5'-taa tat act ggt ctt gta aac c	1.2 kb
	CST39 reverse	5'-ggg tcg gaa ggc tgg gac caa acc	
	^a FOR forward	5'-ccc taa gac tca agg aag aag	
	WAP reverse	5'-ttt tat gta cta cga gcg ca	245
	RED reverse	5'-gtg gtt ggt gga tgt aaa at	199
	NIP reverse	5'-tat ctt acg cac cgg ttt at	299
	REIN reverse	5'-gtg cca tgt acg atc aat aat	375
Cytochrome <i>b</i>	L14724 forward	5'- cga agc ttg ata tga aaa acc atc gtt g	489
	H15149 reverse	5'- aaa ctg cag ccc ctc aga aat gat att tgt cct ca	

^aThe FOR primer was designed from the common sequences of four deer.

Table 2. Capillary electrophoresis primer sequences.

Primer name	Sequence (5' → 3')
RED G*	JOE - GTG GTT GGT GGA TGT AAA AT
NIP G*	JOE - TAT CTT ACG CAC CGG TTT AT
WAP G*	JOE - TTT TAT GTA CTA CGA GCG CA
REIN B*	5-FAM - GTG CCA TGT ACG ATC AAT AAT
Cyt F	CCA TCC AAC ATC TCA GCA TGA TGA AA
CR B*	5-FAM - CCC TCA GAA TGA TAT TTG TCC TCA

injected into the capillary with the module GS STR POP4 (1 ml) F (5 s injection, 15 kV, 60°C, run time 24 min, and filter set F). The primers used in this study are shown in Table 2. The samples were subjected to laser-induced fluorescence, and analyzed with GeneScan 3.1.2 Software.

RESULTS

Characterization of sequence variation

To confirm the D-loop sequences of the deer species, we compared the sequence similarities of the 30 samples. Four additional sequences of the *Cervus* gene were obtained from the NCBI nucleotide data bank (<http://www.ncbi.nlm.nih.gov>) for comparison and verification of our data. A total of 30 DNA sequences were examined along with homologous sequences for the multiplex PCR and capillary electrophoresis, and several tests, including specificity and sensitivity tests, were carried out for multiplex characterization. The sequence differences between the red deer, wapiti and reindeer were relatively small. When the sequences were compared with those registered on Genbank AF016972 and AF016972 (*C. elaphus*) in red deer, AF016979 (*C. elaphus nelsoni*) in wapiti and AF096441 and AF096413 (*R. tarandus groenlandicus*) in reindeer, 99% similarity was obtained. The sika deer sequence showed 98%

similarity with Genbank sequences (accession No. AB186347, AB012371 and AB012373), but the samples of the sika deer showed the same level of similarity. We registered a species each of wapiti (AY970666) and reindeer (AY970667) in the NCBI database. In terms of primer production, this could result in the sequencing of existing red deer and sika deer, as well as of the newly-determined wapiti species and reindeer. We confirmed the antler DNA sequences and the sequences used to analyze the primer design.

Multiplex PCR for antler identification

We amplified 7 primers for 4 antlers and 1 internal control in a single PCR. The 4 antlers and 1 internal control were incorporated in each multiplex PCR. The cytochrome *b* gene was used as an internal size standard, and a 489 bp amplified product was obtained. Deer-specific primer products were not present in the 6 mammal species, but the internal control was detected in all samples. The PCR products derived from each of the antlers were separated by gel electrophoresis, and then identified according to their sizes: 199, 299, 245 and 375 bp from red deer (RED), sika deer (NIP), wapiti (WAP), and reindeer (REIN), respectively (Figure 1). The species-specific and multiplex primers were amplified from deer species

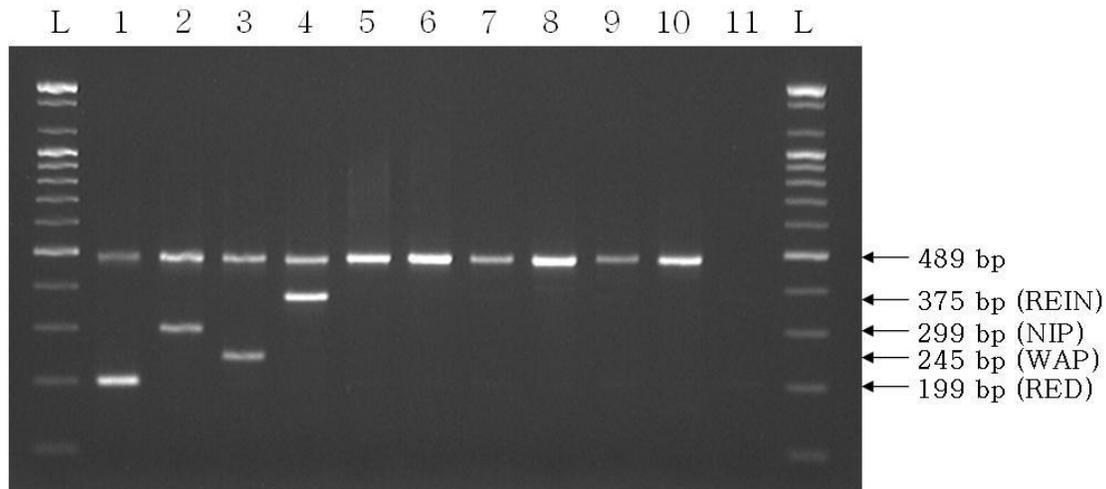


Figure 1. PCR products of species-specific mitochondrial D-loops and cytochrome *b*. 1, red deer; 2, sika deer; 3, wapiti; 4, reindeer; 5, mouse; 6, rat; 7, cat; 8, cow; 9, dog; 10, human; 11, negative control (D.W.); L, 100 bp ladder.

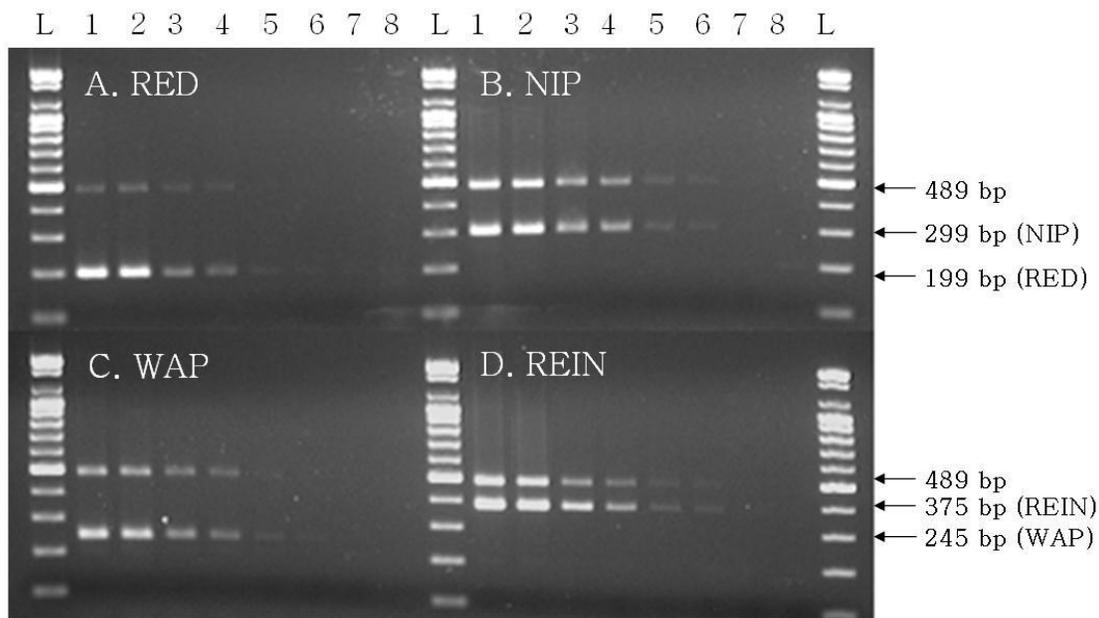


Figure 2. The sensitivity of species-specific mitochondrial D-loops and cytochrome *b*. A, red deer; B, sika deer; C, wapiti; D, reindeer; 1, 1 ng; 2, 500 pg; 3, 100 pg; 4, 50 pg; 5, 10 pg; 6, 5 pg; 7, 1 pg; 8, 0.5 pg; L, 100 bp ladder.

sequences, but were undetectable in the 6 samples of mammalian DNA.

Sensitivity and specificity of mixture detection

To confirm the detection of an optimal PCR reaction for multiplex primers, DNA quantification was performed to assess the quality of the amplification product. Amplification products of an expected specific DNA

fragment size were obtained from 0.5 pg to 1 ng samples (Figure 2). It was confirmed that identification of deer species is possible when at least 50 pg DNA is available; no amplification products were obtained from the 5, 1 or 0.5 pg DNA samples. The sensitivity limit of the internal control using cytochrome *b* was similar to that of the target-specific genes.

The ability to detect DNA concentrations in the mixed and cutting samples could be used as a practical approach. It is often extremely difficult to distinguish

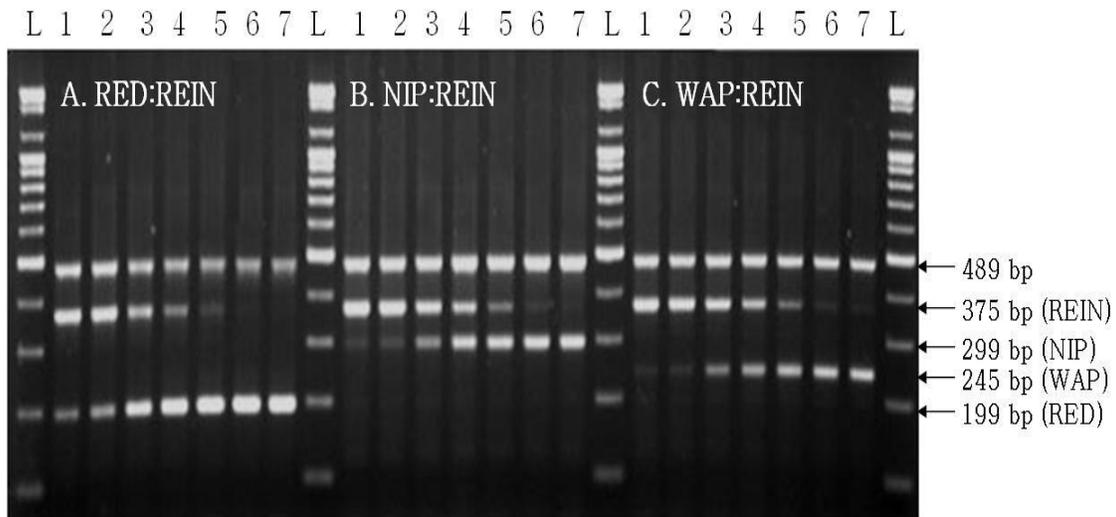


Figure 3. Comparison of amplification patterns from mixed samples. A, red deer (a): reindeer (b). B, sika deer (a): reindeer (b). C, wapiti (a): reindeer (b). 1, a : b = 1:14; 2, a : b = 1:9; 3, a : b = 3:7; 4, a : b = 5:5; 5, a : b = 7:3; 6, a : b = 9:1; 7, a : b = 14:1; L, 100 bp ladder.

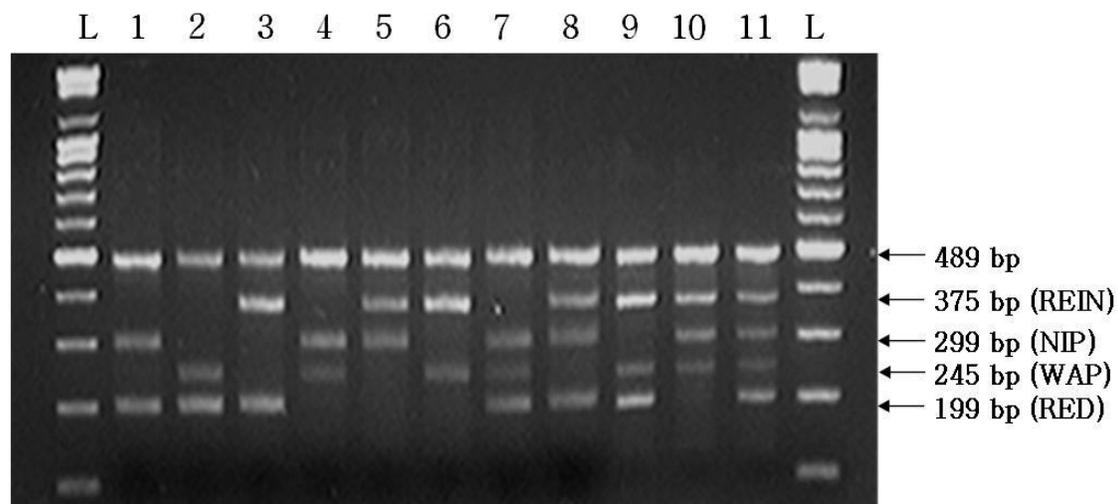


Figure 4. Mixture detection of species-specific mitochondrial D-loops and cytochrome *b*. 1, red deer : sika deer = 1:1; 2, red deer : wapiti = 1:1; 3, red deer : reindeer = 1:1; 4, sika deer : wapiti = 1:1; 5, sika deer : reindeer = 1:1; 6, wapiti : reindeer = 1:1; 7, red deer : sika deer : wapiti = 1:1:1; 8, red deer : sika deer : reindeer = 1:1:1; 9, red deer : wapiti : reindeer = 1:1:1; 10, sika deer : wapiti : reindeer = 1:1:1; 11, red deer : sika deer : wapiti : reindeer = 1:1:1:1; L, 100 bp ladder.

mixed samples on the basis of morphological differences. To ensure that the multiplex reaction for each primer was specific, it was run in reactions with various DNA templates at other reaction rates. When the red deer, sika deer and wapiti samples were mixed with the reindeer samples, sensitivity depended on the mixture rate of each samples. An optimal mixture rate was successfully amplified from 30 to 50% across the whole range (lanes 3 to 5), and from 10 to 93% in the mixture range (Figure 3). We also confirmed the specificity of this mixture against a

range of DNAs mixed at other ratios. Under the same experimental conditions, deer species and internal control DNA could be detected across a range of ratios, from 1:1 to 1:1:1:1 (Figure 4). The amplification of the internal control (489 bp) was not affected in these mixtures, and the specificity of each primer pair was verified. The identification of multiple species in a single polymerase chain reaction (PCR) was found to be highly specific and accurate. The specificity and sensitivity of the multiplex primers indicate their value for use in the detection of

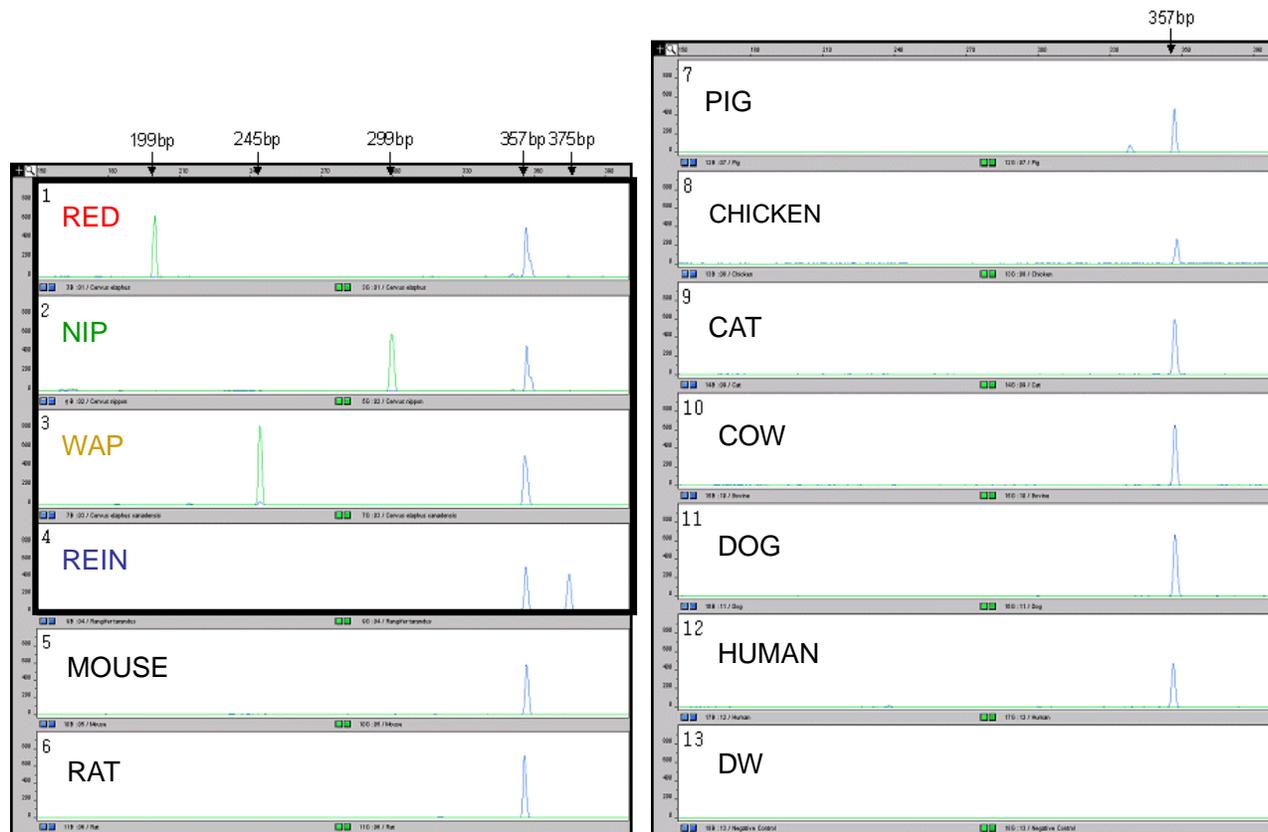


Figure 5. PCR products of species-specific mitochondrial D-loops and cytochrome *b*. 1, Red deer; 2, sika deer; 3, wapiti; 4, reindeer; 5, mouse; 6, rat; 7, pig; 8, chicken; 9, cat; 10, cow; 11, dog; 12, human; 13, negative control (D.W); L, 100 bp ladder.

antler discrimination.

Capillary electrophoresis for antler identification

The specificity of the designed primer for antler identification was reconfirmed by using capillary electrophoresis of the blood and tissue samples of 4 deer antlers and 8 other mammalian DNA samples (mouse, rat, pig, chicken, cat, cow, dog and human). Four reverse primers were labeled with a fluorochrome at the 5' end (JOE or 5-FAM). The modified primers (Cyt F and CR B primers listed in Table 2) described by Kocher et al. (1989) were used as the new internal control for capillary electrophoresis. A 357 bp blue amplified product was obtained in all the samples. The PCR products derived from each of the antlers were separated by capillary electrophoresis, and then identified according to their sizes and color: RED (199 bp, JOE), NIP (299 bp, JOE), WAP (245 bp, JOE) and REIN (375 bp, 5-FAM) (Figure 5). The sizes of the amplified products obtained by gel electrophoresis and capillary electrophoresis were both similar, confirming the suitability of these deer species-

specific primers for differentiating the deer species from each other.

DISCUSSION

The reliability of material detection systems depends on the use of appropriate genes, which should be species-specific and exhibit low heterogeneity within species (Gao et al., 2004). To select a suitable gene for use as a target gene for PCR amplification, we compared the NCBI gene database in order to obtain sequences with sufficient interspecific divergence to produce *Cervus* species-specific products. Four separate groups, red deer, sika deer, wapiti and reindeer were used for the comparison of mitochondrial sequences of the control region. Some researchers who investigated the morphological characteristics such as the adult pelage coloration and immunological data have emphasized that the wapiti has a closer phylogenetic relationship with sika deer than with European red deer (Polziehn and Strobeck, 2002). Inclusion of the sika deer (*C. nippon*) as a sister taxon in the phylogenetic analysis clearly

demonstrated that red deer and wapiti belong to monophyletic groups (Polziehn and Strobeck, 2002). In this study, analysis of phylogenetic relationships revealed that the wapiti is more closely related to the sika deer than to the red deer.

Among the *Cervus* species, the antlers of red deer, sika deer and wapiti are generally used for medicinal purposes. The reindeer is not among the species whose antlers are used for medical prescriptions. It is difficult to identify species accurately based on their morphological classifications in the cutting state. The ability to distinguish deer from cutting material can be important in certain cases, particularly in tissues that are commonly encountered in international trade. The specificity of the multiplex assay was confirmed by performance checks of the system using seven mixed primer combinations from wapiti, red deer, sika deer, reindeer and the internal control. All the expected genes were detected in the mixtures without interference with the PCR reaction, but were undetectable in other mammalian DNA (Figure 1). To validate the SNP multiplex assay, its segregation patterns, detection limits, sample-to-sample reproducibility and species specificity were investigated (Dixon et al., 2005). Thus, we tested a total of 4 individual DNA samples at 8 different concentrations. These results revealed that optimal DNA concentrations were obtained at amounts ranging from 50 pg to 1 ng (Figure 2). The assay was able to detect the 4 deer species only at DNA levels greater than >50 pg. The sensitivity of the tetraplex system is suitable for forensic casework; the species specificity of the assay will be the subject of a developmental validation study (Juusola and Ballantyne, 2005). Furthermore, it was important to test the multiplex system using single source and mixed samples, since such a range of sample types is encountered in forensic casework. Our multiplex assay was a tetraplex system, which included 4 deer-specific genes each, and the specificity of the multiplex was confirmed by performance checks of the system using mixed rate and mixed sample combinations (Figure 3). The duplex (Figure 3) or tetraplex assay (Figure 5) performed well independent, even if other deer samples were included in the admixture.

Single nucleotide polymorphism (SNP) multiplex analysis followed by primer extension has been reported previously. In the reported analysis, a single tube reaction for multiplex PCR was split into 5 tubes for a primer extension assay, and 1 ng reactions were used for the analysis (Inagaki et al., 2004; Dixon et al., 2005). The resulting tetraplex was able to reproducibly identify each of the 4 deer species when it contained one or more of the mixed deer samples. The amplified products obtained by gel electrophoresis (Figure 1) and capillary electrophoresis (Figure 5) were both similar in size, confirming the suitability of the deer species-specific primers for differentiation between species. The specificity of detection was very high; therefore, we

believe that the tissue-specific PCR assay can accurately distinguish between deer species and determine the sizes of the PCR products in mixed samples, which can be analyzed in a single tube.

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