J Korean Soc Appl Biol Chem (2015) 58(1):61–66
DOI 10.1007/s13765-015-0005-2

Online ISSN 2234-344X
Print ISSN 1738-2203

ARTICLE

Development of a PCR-based assay to differentiate *Cervus elaphus sibiricus* from *Cervus* antlers

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Received: 8 August 2014 / Accepted: 9 November 2014 / Published online: 30 January 2015
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Abstract Deer antlers (Cervi Parvum Cornu) are reported to possess various pharmacological properties, including anti-infective, anti-arthritic, anti-allergic, and anti-endometriotic properties, and are credited with reversing memory impairment. To determine the global distribution of *Cervus* subspecies by analyzing antlers, 166 samples of *Cervus* subspecies antlers were collected from Russia, Canada, China, New Zealand, and Korea. The respective deer subspecies were identified by amplifying the D-loop region of mitochondrial DNA isolated from the antler samples. On the basis of the mitochondrial DNA sequence, a *C. elaphus sibiricus*-specific primer was developed that differentiates *C. e. sibiricus* from four *C. e.* subspecies (*C. e. manitobensis*, *C. e. nelsoni*, *C. e. canadensis*, and *C. e. bactrianus*), *C. elaphus*, and *C. nippon*. This was confirmed using agarose gel electrophoresis. This primer set produced a specific 396-bp fragment that amplified only *C. e. sibiricus* in antler samples. A 468-bp fragment derived from the cytochrome *b* gene was used as the internal control to verify the success of amplification in all samples. This method may assist in rapid and effective differentiation between products from *Cervus* species and other deer products.

Keywords Agarose gel electrophoresis · *Cervus* antlers · *Cervus elaphus sibiricus* · Differentiation · D-loop region · Mitochondrial DNA

Electronic supplementary material The online version of this article (doi:10.1007/s13765-015-0005-2) contains supplementary material, which is available to authorized users.

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Introduction

Cervi Parvum Cornu is a well-known crude drug derived from horizontal slices of the velvet-covered antlers of male *Cervus* ssp. deer (Kim et al. 2012c). Various results, including memory improvement (Lee et al. 2010) and anti-infective (Dai et al. 2011), anti-arthritic (Kim et al. 2004), anti-allergic (Kuo et al. 2012), and anti-endometriotic (Kim et al. 2012b) effects, have been reported after use of this product. It has been widely used as an ingredient in functional foods.

To date, two types of Cervi Parvum Cornu, derived from *C. nippon* (sika deer) and *C. elaphus* (red deer), have been described in the Korean Herbal Pharmacopoeia (Han et al. 1994). The efficacy and price of antlers vary according to the grade and source of the antler slices (Kim et al. 2012c). The grade and origin of antler slices also affect the price of functional foods containing them. In Korea, antlers are mainly imported from Russia, New Zealand, and China. In Korean markets, Russian antlers are considered to be of particularly good quality and command the highest prices. Merchants may lie about the origin of antlers to charge

higher prices. Therefore, consumers should be cautious about buying deer antlers or related products. It is difficult to distinguish morphological differences in origin or subspecies of antlers. For this reason, it is necessary to develop a method that can distinguish Russian antlers from other *Cervus* antlers.

The quality control of deer products depends primarily on macroscopic identification, microscopic examination, and physical and chemical analyses. However, deer products cannot always be evaluated using these methods because of the different technical processes used to prepare the products (Zha et al. 2011). The use of PCR techniques to identify deer products has been extensively investigated. The PCR approach tends to be more specific, sensitive, and applicable even to heat-processed products (Bottero et al. 2003; Dalmasso et al. 2004). The D-loop region of mitochondrial DNA (mtDNA) has been used to classify deer subspecies (Polziehn et al. 1998; Polziehn and Strobeck 1998, 2002). In our previous studies, we used sequences from the D-loop region to differentiate between deer species and *Cervus* antlers (Kim et al. 2012a, c).

The goal of the present study was to develop a PCR method to differentiate the most expensive Russian antlers from other antlers in the Korean herbal market. First, we performed subspecies identification of 166 *Cervus* antler samples collected from China, New Zealand, Canada, Korea, and Russia by amplifying the D-loop region of the mtDNA. All Russian antlers were confirmed to be *Cervus elaphus sibiricus*. Finally, we developed a PCR-based assay to differentiate between *C. e. sibiricus* antlers and other *Cervus* antlers.

Table 1 Details of the 166 *Cervus* antler samples used in this study

Collection area	Collection location	Collection date	Number of antlers
China	Jilin Province	2008.01	19
	Xinjiang	2008.01	21
New Zealand	Auckland	2008.01	9
	Christchurch	2008.01	17
Canada	Vancouver	2008.02	10
	Calgary	2008.02	10
	Edmonton	2008.02	20
Korea	Gyeongju	2008.01	7
	Daegu	2008.01	3
	Namwon	2008.01	8
	Yongin	2008.01	13
Russia	Altai Republic	2008.07	29
Total			166

Table 2 Primer sequences used in this study

Primer	Oligonucleotide sequence (5´ → 3´)
CST2[a]	TAA TAT ACT GGT CTT GTA AAC C
CST39[a]	GGG TCG GAA GGC TGG GAC CAA ACC
SF4	CCT ACT AAT TAC ACA GCA AAA CAC A
SR10	TAG CTA CCC CCA CGG TC
L14724-tm58[b]	TTG ATA TGA AAA ACC ATC GTT G
H15149-tm58[b]	TCA GAA ATG ATA TTT GTC CTC A

[a] Primer sequences according to the original method (Polziehn et al. 1998)

[b] Primer sequences according to the original method (Kim et al. 2012c)

Materials and methods

Sample collection

We collected 166 *Cervus* antler samples from various processing plants in China, New Zealand, Canada, Korea, and Russia (Table 1). Voucher samples for the antlers were deposited with the Korea Institute of Oriental Medicine.

DNA extraction, amplification, and sequencing

DNA was extracted from the antler slices using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. Purity of the DNA was confirmed using an ND-1000 spectrophotometer (NanoDrop Tech., USA). Primers CST2 and CST39 (Table 2) were used to amplify the D-loop region of mtDNA from the extracted DNA (Polziehn et al. 1998). The PCR conditions and cloning method were described in a previous study (Kim et al. 2012c). DNA was sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems, USA). The sequences were aligned using DNASIS MAX version 2.05 (MiraiBio, USA) and BioEdit Sequence Alignment Editor version 7.0.9.0 (Ibis Biosciences, USA).

Primer design

After sequence alignment of the D-loop region, we designed a 25-mer SF4 primer (forward primer) and 17-mer SR10 primer (reverse primer) to differentiate between *C. e. sibiricus* and other *Cervus* species antlers. Primer locations and sequences are shown in Fig. 1 and Table 2, respectively.

C. e. sibiricus-specific PCR by agarose gel electrophoresis

Primer sets SF4/SR10 and L14724-tm58/H15149-tm58 (Table 2) were used to amplify gene sequences in the mitochondrial D-loop and cytochrome *b* regions,

respectively. PCR amplifications were performed in a 20-μL volume containing 5 U/μL GoTaq® Hot Start polymerase 0.2 μL (Promega, USA), 4 μL of 5× Green GoTaq® Flexi buffer (Promega, USA), 1 μL of dNTP mixture (Takara Bio Inc., Japan), 1.6 μL of 25 mM $MgCl_2$ solution, 1 μL of 20 ng/μL template DNA, 2.4 μL of 10 pmol primer (SF4/SR10), and 0.7 μL of primer set L14724-tm58/H15149-tm58 (Table 2). The PCR conditions were: initial denaturation at 95 °C for 4 min; 21 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 6 min. PCR was conducted using a C1000™ Thermal Cycler (Bio-Rad, USA). Amplified products were separated in a 2 % (w/v) agarose gel with 1 μL LoadingSTAR (Dynebio, Korea) in 0.5× TBE buffer (45 mM Tris–borate and 1 mM EDTA; pH 8.0) and were analyzed using a U:Genius (Syngene, UK).

Results

Identification of *Cervus* antlers using mitochondrial D-loop sequence

To confirm that the 166 antlers belonged to *Cervus* subspecies, we compared the homology of D-loop sequences of the amplified fragments with known nucleotide sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

Most samples showed 98–99 % identity with the GenBank sequences, except for the 19 samples from Jilin Province, China, which showed 95–96 % identity with *C. nippon* (accession number AF016974). These samples had similar nucleotide sequences and were divided into two groups; the two identified sequences were deposited in GenBank as *C. nippon* NIP1 (KF141944) and NIP2 (KF141945).

The 20 samples collected from Xinjiang and other areas in China showed 99 % identity with *C. e. bactrianus* (AF296822). Two new sequences of *C. e. bactrianus* were found and deposited as *C. e. bactrianus* BAT1 (KF141938) and BAT2 (KF141939). Both sequences showed three base insertions and two base variations compared with AF296822. *C. e. bactrianus* BAT2 (KF141939) showed one additional sequence variation. One Xinjiang sample was similar to *C. elaphus* (AF016973) (99 % identity).

Almost all of the samples collected in New Zealand were identified as *C. elaphus*. Twenty-five samples showed 98–99 % identity with *C. elaphus* (AF016973 = 23 samples, Y08207 = 2 samples). Four new *C. elaphus* sequences were found and were deposited in GenBank as *C. elaphus* ELA1–ELA4 (KF153093–96). All newly deposited sequences showed one base insertion, one base deletion, and three base variations compared with AF016973. One sample had 99 % identity with *C. e. nelsoni* (AF016965) and was identified as *C. e. nelsoni*.

The Canadian samples represented three deer subspecies, including 24 Canadian samples that showed 99 % identity with *C. e. nelsoni* (AF016962 = 15 samples, AF016965 = 9 samples). We found two new sequences of *C. e. nelsoni*, deposited in GenBank as *C. e. nelsoni* NEL1 (KF153097) and NEL2 (KF153098). Each new sequence showed one base deletion and variation compared with AF016962. In addition, 13 Canadian samples were identified as *C. e. manitobensis* (AF016955 = 3 samples; AF016957 = 9 samples; AF005199 = 1 sample). Two new sequences of *C. e. manitobensis* were found and deposited in GenBank as *C. e. manitobensis* MAN1 and MAN2 (KF153091 and KF153092). Both sequences showed one base deletion and one base variation compared with AF016957. *C. e. manitobensis* MAN2 (KF153092) showed two additional sequence variations. The final three samples collected in Canada showed 99 % identity with *C. e. canadensis* (AY970666). Two of these showed three base variations compared with AY970666, and that sequence was deposited as a new *C. e. canadensis* sequence (KF141943).

The Korean samples also represented three subspecies of deer, including 10 samples that showed 99 % identity with *C. elaphus* (AF016973), 11 samples showed 99–100 % identity with *C. e. canadensis* (AY970666), and 10 samples that showed 99 % identity with *C. e. nelsoni* (AF016962, AF016965, and AF016966). We found two new sequences of *C. e. nelsoni* in the Korean samples and deposited these in GenBank as *C. e. nelsoni* NEL3 (KF153099) and NEL4 (KF153100). These new sequences had one more base deletion than *C. e. nelsoni* NEL1 (KF153097) and NEL2 (KF153098).

Finally, 29 Russian samples showed 99 % identity with *C. e. sibiricus* (AF058371). Two new sequences of *C. e. sibiricus* were deposited in GenBank as *C. e. sibiricus* SIB1 and SIB2 (KF141940 and KF141941). Both sequences showed one base deletion compared with AF058371. *C. e. sibiricus* SIB2 showed one additional base deletion and seven base variations. All the results of the identification of 166 *Cervus* subspecies antler samples are summarized in Table 3. The details of BLAST searches for all 166 *Cervus* antlers are provided in Supplementary Table 1.

Differentiation of *C. e. sibiricus* using electrophoresis-based PCR assay

We observed a difference in the D-loop sequences, which we used to design a primer set (SF4 and SR10). This primer set produced a specific 396-bp fragment that amplified only *C. e. sibiricus* by agarose gel electrophoresis, allowing it to

Table 3 Identification of 166 *Cervus* antler samples by using mitochondrial D-loop sequence

Collection area	Subspecies	Number of antlers	Associated GenBank accession no.
China	*C. nippon*	19	KF141944, KF141945
	C. e. bactrianus	20	AF296822, KF141938, KF141939
	C. elaphus	1	AF016973
New Zealand	*C. elaphus*	25	AF016973, Y08207, KF153093, KF153094, KF153095, KF153096
	C. e. nelsoni	1	AF016965
Canada	*C. e. nelsoni*	24	AF016962, AF016965, KF153097, KF153098
	C. e. manitobensis	13	AF016955, AF016957, AF005199, KF153091, KF153092
	C. e. canadensis	3	AY970666, KF141943
Korea	*C. elaphus*	10	AF016973
	C. e. canadensis	11	AY970666
	C. e. nelsoni	10	AF016962, AF016965, AF016966, KF153099, KF153100
Russia	*C. e. sibiricus*	29	AF058371, KF141940, KF141941
Total		166	

[a] Underlined GenBank accession numbers have been registered according to our findings in this study

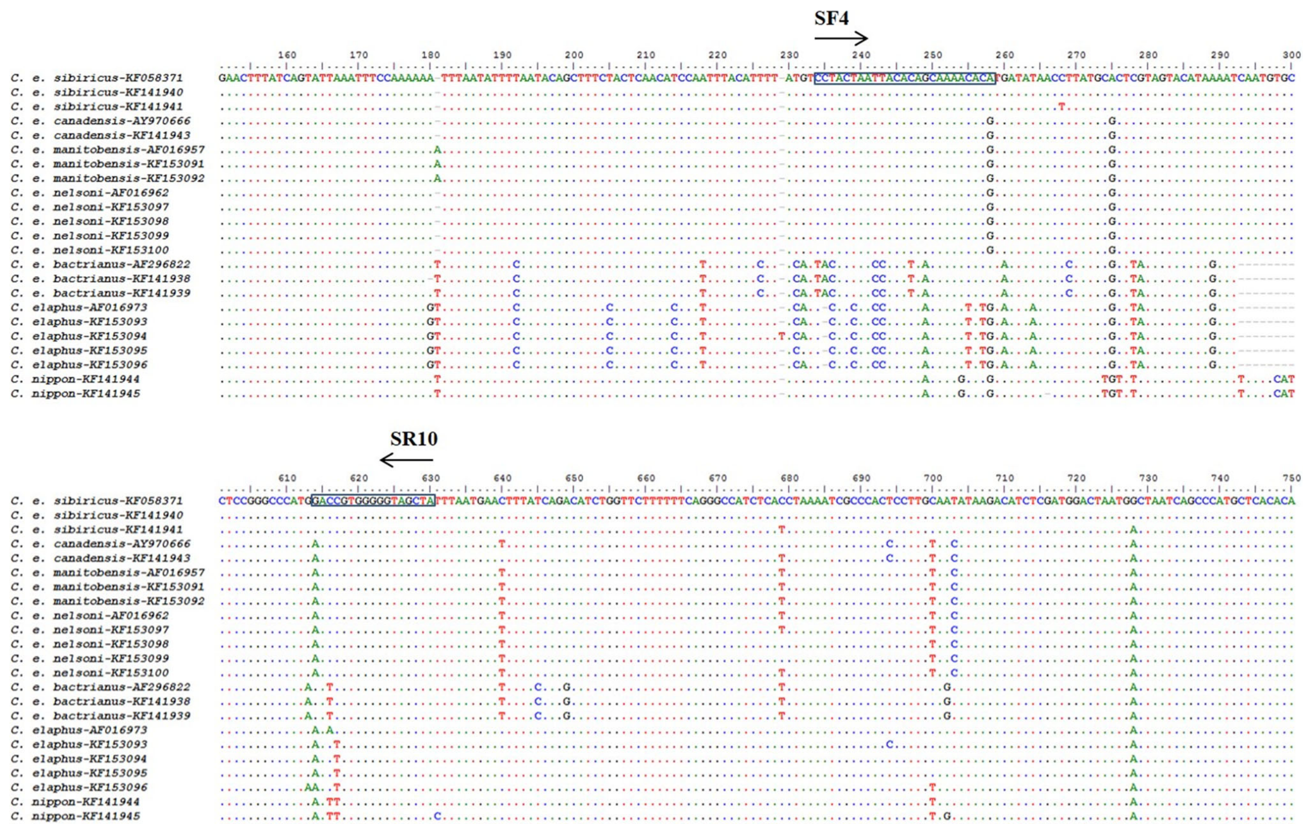

Fig. 1 Partial sequences of the mitochondrial DNA (mtDNA) control region used to distinguish *C. e. sibiricus* from other *Cervus* subspecies in antler samples. *Dots* represent identical bases to the first sequence. Primer sets are marked by *blue square boxes*; the sequences are presented in Table 2

be distinguished from other Russian antler samples (Lanes 1 and 2 in Fig. 2). A 468-bp fragment derived from the cytochrome *b* gene was used as the internal control to verify the success of amplification in all samples (Fig. 2). These primers were reconfirmed in all 166 samples, including 29 Russian samples (Supplementary Fig. 1).

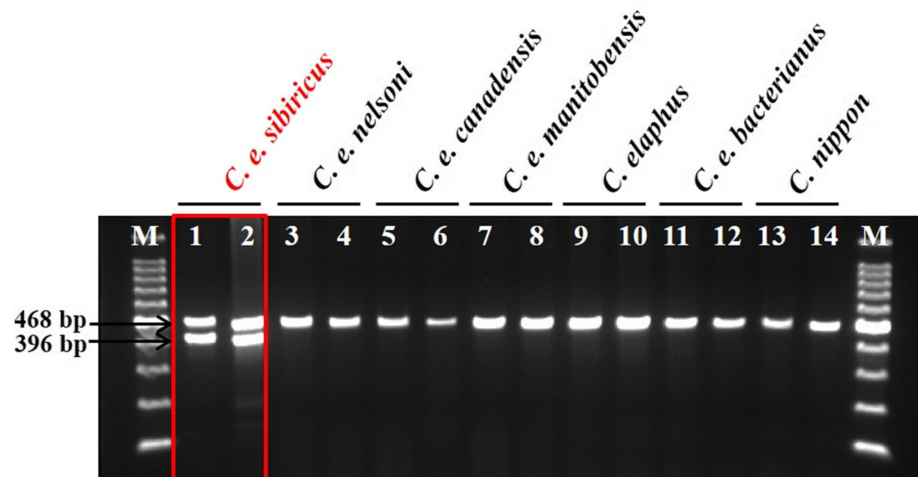

Fig. 2 PCR products generated with *C. e. sibiricus*-specific primers. *M* = 100 bp on a DNA size standard ladder

Thus, the DNA sequencing analysis verified that all antler samples collected from Russia belonged to *C. e. sibiricus*.

Discussion

The consumption of antlers in Korea has been increasing steadily each year, concomitant with economic growth, and imports of antlers have also increased. *Cervus* antlers are primarily imported to Korea from Russia, New Zealand, and China. In Korean markets, the most expensive deer antlers are imported from Russia. However, *Rangifer* antlers, derived from reindeer (*Rangifer tarandus*), and *Cervus* antlers produced in North America are sold illegally in herbal markets as Russian *Cervus* antlers (Yoo et al. 2010). Both males and females of the genus *Rangifer* have antlers, whereas only males of the genus *Cervus* have antlers (Shah et al. 2008). *Rangifer* antlers are not considered viable drug material because their ash content does not meet the specified minimum value (Korea Food & Drug Administration 2014; Shim et al. 2011). Since 2001, the import of North American deer antlers infected with chronic wasting disease has been banned in Korea.

In the Korean Herbal Pharmacopoeia and Chinese Pharmacopoeia, the definition of the chemical standards of Cervi Parvum Cornu is not complete. Only the appearance, identification, loss of material during drying, and ash content are specified (Chinese Pharmacopoeia Commission 2010; Korea Food & Drug Administration 2014). It remains difficult to identify subspecies using morphological characteristics and sensory evaluation tests. Morphological distinction is particularly difficult when antlers are sold as cuttings. Several studies have attempted to establish standards to evaluate the quality of Cervi Parvum Cornu, and the amounts of free amino acids, ganglioside, and glycosaminoglycan were proposed as the standard (Hong et al. 1991, 1993; Sunwoo and Sim 2001). Despite such efforts, the criteria to evaluate the quality of antlers remain insufficient, and studies of the pharmacological properties of antlers from different subspecies and regions have not been completed.

Therefore, it is necessary to develop a method that precisely differentiates the region of production and the subspecies of deer antlers. The most effective means of determining the subspecies is analysis of the nucleotide sequences of specific gene regions. However, this procedure is costly and it is challenging to obtain results quickly using this method. If a large number of samples must be analyzed, PCR-based methods are more practical and cost-effective.

In recent years, molecular methods, such as PCR-based assays, have been widely used to differentiate between animal species or subspecies. Oh et al. (2000) conducted assays of Korean beef cattle and Holsteins based on SNP allele differentiation of genes related to coat color. Kim et al. (2012c) and Shim et al. (2011) reported a PCR-based assay for differentiating *Rangifer* and *Cervus* antlers. Until recently, little research had been conducted on Russian deer. Baranova et al. (2012) investigated the genetic diversity of *R. tarandus* inhabiting the European part of Russia. ZvychaÌnaia et al. (2011) conducted polymorphism analysis of mtDNA of the Siberian roe deer (*Capreolus pygargus*) from 23 regions of Russia and Kazakhstan.

To the best of our knowledge, the present study is the first to focus on identifying the subspecies of deer antlers produced in Russia. We developed a rapid and simple method using specific primers to distinguish *C. e. sibiricus* from other *Cervus* subspecies by analyzing antler samples, exploiting differences in the nucleotide sequences of the mtDNA. Agarose gel electrophoresis was used to confirm the *C. e. sibiricus*-specific primers. Our technique provides more accurate and simple subspecies identification. It may

be helpful for quality control and detection of Cervi Parvum Cornu and other products derived from deer.

Acknowledgments This research was supported by a Grant (07092KFDA309) from the Korea Food and Drug Administration and a Grant (K14101) from the Korea Institute of Oriental Medicine.

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