BIOCHIP JOURNAL, Vol. 3, No. 3, 261-265, September 2009

Diagnostic Polymorphisms in the Mitochondrial DNA Allow Discrimination among *Cervus elaphus* Species

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Accepted 19 August 2009

Abstract

Velvet antlers from *Cervus elaphus* species are one of most famous and commonly used medicinal materials in traditional oriental medicine. In this study, the variation in DNA sequences of the mitochondrial ATPase8 and cytochrome-c oxidase I (COI) genes of *Cervus elaphus* from China, the Republic of Altai, and Canada were evaluated. In addition, the sequence variation among Sika deer, Rein deer and *Cervus elaphus* species was also evaluated. Although the sequences of deer from the Republic of Altai and Canada were very similar, polymorphisms that were conserved in each species were observed in the ATPase8 and COI genes. Therefore, these polymorphic markers could be used to distinguish *Cervus elaphus* antlers from different locations.

Keywords: *Cervus elaphus*, Velvet antler, Deer, Polymorphisms, Mitochondrial DNA

Introduction

Velvet antlers refer to the soft growing tissues found in the antlers of deer. Velvet antlers are commonly used for the treatment of various diseases in oriental medicine, and commercially farmed deer antlers have emerged over the last few decades. Deer antlers, which are rich in keratin, have been used for the treatment of neurosis, enrichment of vital energy, nursing the blood, strengthening the kidney, treatment of rheumatoid arthritis (RA) and prolonging life for thousands of years. It has been demonstrated that protein analysis of the keratin in deer antlers may be used as a standardization index, because keratin is common to each specific species. In addition, proteome analysis of red deer antlers has shown that several cell growth or signaling related proteins are expressed exclusively in the growing tip, which is believed to be the best part of the antler for traditional medicinal purposes[1]. Recently, many studies have been conducted to evaluate the pharmacological effects of the components of deer antlers, and the results of these studies have demonstrated that these components have anti-inflammatory effects, anti-whiplash effects that occur via anti-stress activities, and anti-aging activities[2].

The velvet antler trade is a large business, and 80% of the velvet antlers produced worldwide are consumed in Korea. Most velvet antlers sold in Korea are imported from China, New Zealand, Canada and the Republic of Altai. Although Canadian antlers can no longer be imported to Korea due to an outbreak of chronic wasting disease (CWD) in Canada in 1996, it has been reported that Canadian antlers are smuggled and distributed in the market. In addition, although there have been no reports of differences in the ingredients or medicinal effects of antlers from different sources, Korean consumers tend to believe that antlers from the Republic of Altai have the most medicinal effects. For this reason, antlers from the Republic of Altai are 2 to 3 times more expensive than antlers from China or New Zealand, which has resulted in the illegal practice of disguising the origin of antlers. Therefore, a test to distinguish antler based on their origin is essential to ensure the healthy development of the herbal industry.

Traditionally, the authentication of Korean herbs has relied on morphological and histological inspection. However, antlers from the Republic of Altai, New Zealand, China and Canada are very similar morphologically. Furthermore, many antler products are sold in shredded slices, which make it difficult or impossible to identify them using morphological and histological methods.

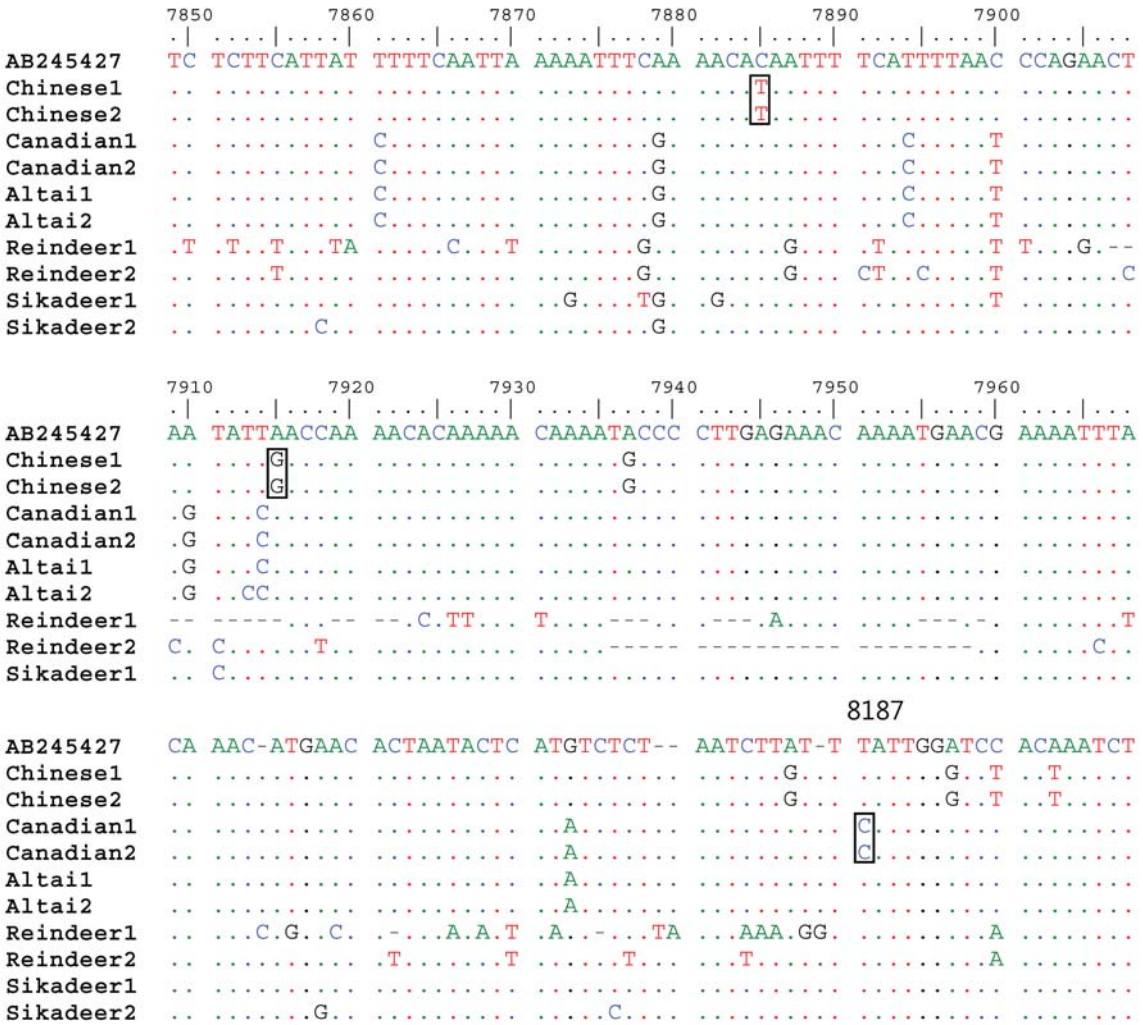

Figure 1. Sequence alignment of the ATPase8 region. Dashes indicate indel sites. Dots denote identity with nucleotides of AB245427.

Mitochondrial DNA (mtDNA) has been widely used in phylogenetic studies of animals because it evolves much more rapidly than nuclear DNA, which results in the accumulation of differences between closely related species[3]. Sequence divergence is much higher among species than within species, and mtDNA genealogies generally capture biological discontinuities recognized by taxonomists as species. The cytochrome-c oxidase I (COI) gene is present in all animals, and sequence comparisons are straightforward because insertions and deletions are rare. In addition, congeneric species of animals regularly possess substantial sequence divergence in their COI genes. For example, it has been reported that more than 98% of species pairs showed greater than 2% sequence divergence in COI genes[4]. Therefore, the COI gene, which has also been referred to as the DNA barcode[5], has been used to identify species of birds[6], fungi[7] and tropical Lepidoptera[8].

In addition, Wada and Yokohama (Wada and Yokohama., 2004) studied the divergence of mitochondrial DNA sequences in Yeso Sika deer and found that ATPase8 had the highest value of divergence among of the following regions: NADH dehydrogenase subunits (ND1, ND2, ND3, ND4L, ND4, ND5 and ND6), cytochrome c oxidase subunits (CO I and CO III), ATP synthase subunits (ATPase8 and ATPase6) and cytochrome b.

Therefore, in this study, the COI and ATPase8 genes were evaluated for specific polymorphic nucleotides that could be used to distinguish deer from different locations.

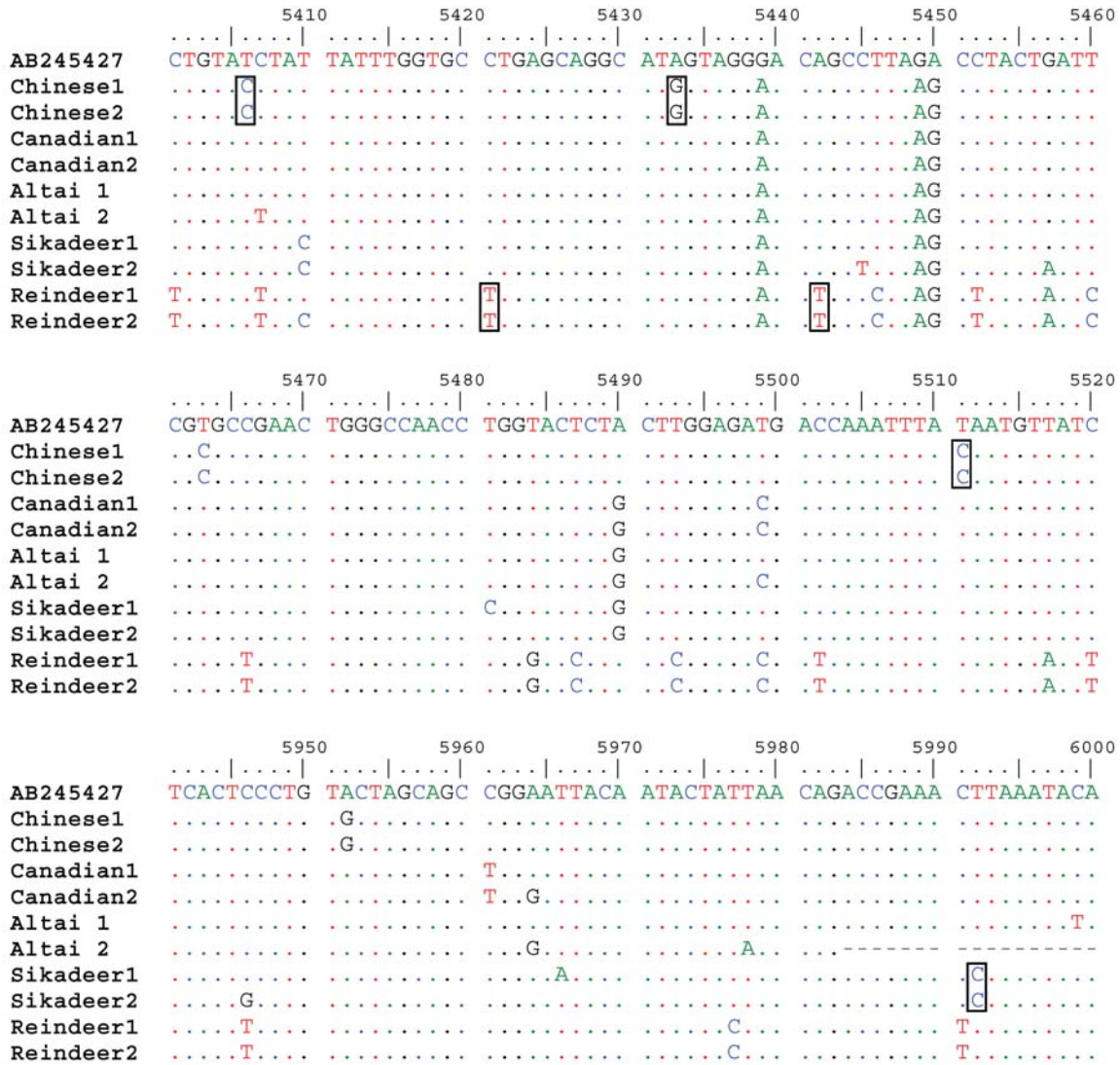

Figure 2. Sequence alignment of the COI region. Dashes indicate indel sites. Dots denote identity with nucleotides of AB245427.

Results and Discussion

Following PCR amplification, amplicons from at least 5 deer from the Republic of Altai, China and Canada were sequenced and aligned to identify a DNA segment with sufficient population to population variation to enable their discrimination. In addition, samples obtained from Sika and Rein deer were also sequenced. As shown Figure 1, all sequences were aligned and compared to the mtDNA full sequence of *Cervus elaphus* (AB245427). The nucleotide sequences of deer from the Republic of Altai and Canada were very similar, however, they could be distinguished by one unique substitution at site 8187 of the reference sequence in the ATPase 8 region (Figure 1 and Table 1), with only Canadian deer having a C nucleotide at that site. And the nucleotide in all of 53 Altai deer was conserved at that site (Data not shown).

In addition, 2 unique variable sites in Chinese deer were observed at sites 7885, and 7915 of the reference sequence in the ATPase 8 region. Additionally, when the COI genes were evaluated, Chinese deer were found to have polymorphic nucleotides at sites 5406, 5433 and 5511, Sika deer were found to have polymorphic nucleotides at site 5992 and Rein deer were found to have two unique nucleotides at sites 5421 and 5442 (Figure 2 and Table 1).

In the phylogenetic tree constructed using ATPase8 sequences, deer from the Republic of Altai and Cana-

Table 1. Polymorphic nucleotides in ATPase8 and COI regions among Rein deer, Sika deer and Cervus elaphus species from Altai, Canadian, and Chinese.

Region	Population	Sample number	Position*	Specific nucleotide	Reference sequence
ATPase8	Chinese	10	7885 7915	T G	AB245427
	Canadian	7	8187	C	
CO1	Chinese	8	5406 5433 5511	C G C	
	Sika deer	5	5992	C	
	Rein deer	14	5421 5442	T T	

* Position is the site of a reference sequence AB245427.

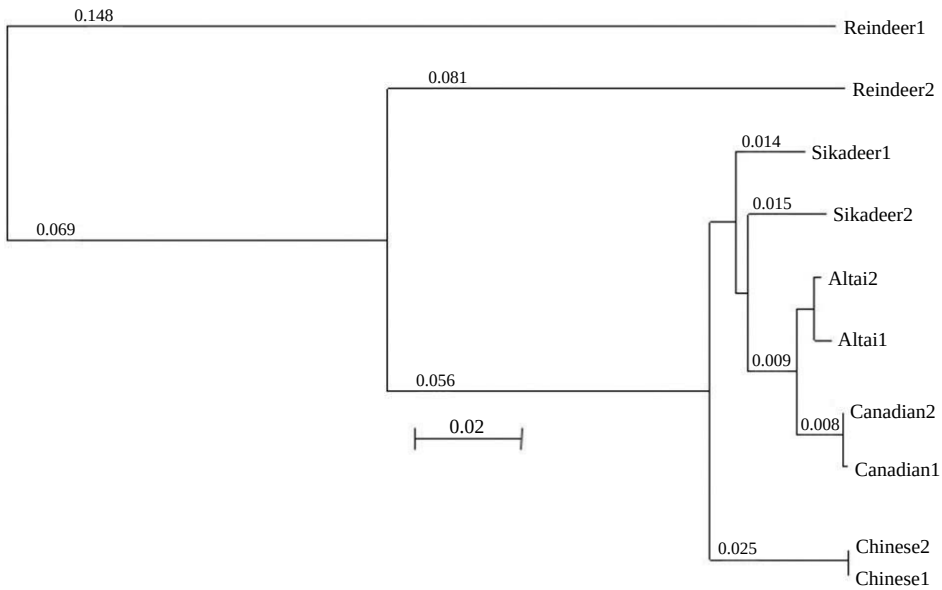

Figure 3. Phylogenetic relationships among Rein deer, Sika deer and Cervus elaphus species from the Republic of Altai, Canada and China using sequence data from the ATPase8 region of mtDNA. The tree shown was created using the neighbor-joining method.

dian deer formed a cluster (Figure 3). It has been reported that Altai deer are primarily *Cervus elaphus sibericus* (Siberian wapiti) and Canadian deer are *Cervus elaphus nelsoni*, *Cervus elaphus roosevelti*, *Cervus elaphus maitobensis* and primarily *Cervus elaphus nannodes* (known as North American wapiti)[10]. Taken together, the results of this study confirmed that deer from the Republic of Altai and Canada were wapiti species and that Chinese and the New Zealand deer populations were red deer species, which is similar to the results of a study[10].

The most important goal of this study was to determine if velvet antlers from deer populations in the Republic of Altai and Canada could be distinguished from those of other populations because antlers from the Republic of Altai are more expensive than other antlers and Canadian velvet antlers have been banned in Korea. By 1996, CWD was first detected in Canada's farmed elk, and soon thereafter in the US elk industry CWD-infected ranched elk have been discovered in several other US states[11,12] and in South Korea and raising international awareness and concern regarding CWD. Although human susceptibility to CWD is still unclear, it was reported CWD could transmitted to livestock such as cattle[13]. So it is important to prevent distribution or consuming of antler or venison from the CWD-infected era.

Conclusions

This study clearly showed that the use of mtDNA from the ATPase8 and COI regions could be used to distinguish deer populations based on their geographic

origins. Therefore, this method may be useful for determination of a deer's subspecies or origins; however, further studies using larger samples sizes should be conducted to confirm these findings.

Materials and Methods

Sample Selection and DNA Extraction

Authentic antler samples were obtained from the PuriMed Co Ltd. (Seoul, Korea) or Jesson Trading Co Ltd. (Seoul, Korea). Genomic DNA was then extracted from deer antlers using an i-genomic CTB DNA extraction mini kit (iNtRON Biotechnology, Inc. Seongnam, Korea). Briefly, 0.05 g of antler was ground to fine powder using a Precellys 24 bead-based homogenizer (Stretton Scientific, Stretton, UK), after which the extraction was performed according to the manufacturer's instructions for bone. Next, the DNA was eluted in 50-100 μL of sterile deionized water, and its concentration was determined using a NanoDrop (NP-1000) Spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE).

PCR Amplification of Each Gene Region

The following COI and ATPase8 primers, which were designed by modifying the primers used by Wada and Yohohama, were used in this study (Wada and Yokohama., 2004):

ATP8-S: GGA CGC AAT TCC AGG CCG CCT
ATP8-AS: TGCTCACAGGGGAATGGCTATG
CO1-S: TTCAATCTACTTCTCCCGCCGC
CO1-AS: AGGATAATAGCCGGTAGGATTG

Isolation and Sequencing of PCR Products

Each amplified product was separated on 1.5% low melting agarose gel (Duchefa biochemie B.V. Haarlem, Nederland) and then extracted using a Mega quick spin™ PCR & agarose gel extraction kit (iNtRON Biotechnology, Inc. Seongnam, Korea). The isolated PCR products were then sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA) from COSMO Co Ltd. (Seoul, Korea).

Data Analysis

We compared the sequence data generated in this study to a published sequence of red deer mtDNA full sequences (GeneBank accession number: AB245427). The sequences were aligned using BioEdit version 5.0.9 [BioEdit (URL: http://www.mbio.ncsu.edu/BioEdit/BioEdit.html)]. In addition, a phylogenetic tree was constructed via the neighbor-joining method[9] using Clustal X (v 1.81).

Acknowledgements

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. 2009-0063466) and a grant from the Small & Medium Business Administration (S1015952).

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