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Molecular Discrimination of *Cervidae* Antlers and *Rangifer* Antlers

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***Cervi Parvum Cornu* is widely used as a hemopoietic, tonifying, growth-promoting, cardiotonic, and immuno-modulating agent in Korea. In order to develop the quality control method of *Cervi Parvum Cornu* by the identification of the biological source or origin, the molecular approach was applied using PCR (polymerase chain reaction) and PCR-RFLP (PCR-restriction fragment length polymorphism) analysis. In the PCR analysis of the mitochondrial 12S rRNA gene and cytochrome b gene regions, no distinctive DNA bands from *Cervidae* (deer) antlers and *Rangifer* (reindeer) antlers were observed. However, when the amplified products in the mitochondrial cytochrome b gene region were subjected to restriction digestion with *Taq*I, *Cervidae* antlers showed an undigested state of 380 bp band, differently from two bands of 230 bp and 150 bp from *Rangifer* antlers. Based on this finding, the base sequences of amplified PCR products in the range of mitochondrial cytochrome b gene from *Cervidae* antlers and *Rangifer* antlers were determined and subjected to restriction analysis by various endonucleases. The results showed that antlers from *Rangifer* species could be simply discriminated with other antlers from 8 *Cervidae* species (Chinese deer, Russian deer, Hong Kong deer, New Zealand deer, Kazakhstan deer, elk, red deer and Sika deer) by PCR-RFLP analysis using *Alu*I, *Hae*III, *Hpa*II or *Sau*3AI(*Mbo*I) as well as *Taq*I in the range of the mitochondrial cytochrome b gene.**

Keywords: *Cervi Parvum Cornu*, *Cervidae*, *Rangifer*, PCR-RFLP, Deer antler

Introduction

Cervi Parvum Cornu is a shady-dried antler newly grown from male *Cervidae* in the spring. In the Orient, it has been widely used as a vitalizing, tonifying, hemopoietic and strengthening agent for debilitation persons, as described in old medicinal books for oriental drugs (Han *et al.*, 1994). In fact, it has already been revealed that *Cervi Parvum Cornu* has some pharmacological activities. These include hemopoietic, tonifying, growth-promoting, cardiotonic and immuno-modulating effects (Yook *et al.*, 1998).

Until now, two kinds of *Cervi Parvum Cornu* from *Cervus nippon* and *Cervus elaphus* were described in Pharmacoepia of Oriental countries (Han *et al.*, 1994). The major components in *Cervi Parvum Cornu* are known to be collagen, chondroitin sulfate, ganglioside, acidic muco-polysaccharide, and a lot of calcium (Han and Jhon, 1994; Han *et al.*, 1994; Yook *et al.*, 1998). However, there are some fluctuations in the amount of these components in different antler parts, so-called upper part, middle part and lower part.

The amount of *Cervi Parvum Cornu* consumed annually in Korea reaches to 106 ton. This is as much as 80~90% of the total world consumption (http://magres.ewha.ac.kr/~gjjhon/deer.htm). Most of *Cervi Parvum Cornu* was supplied from China and Russia, but recently the import of *Cervi Parvum Cornu* from New Zealand and other countries has been seriously considered due to the limited supply.

The quality control of *Cervi Parvum Cornu* has been mainly dependent upon direct examination of the shape by the unaided eye. Some chemical analytic methods were also reported for the determination of calcium content, amino acid composition, or the amount of pharmacologically-active gangliosides (Han and Jhon, 1994). But, the application of those chemical methods is restricted in evaluating *Cervi Parvum Cornu* without direct eye examination.

Recently, detection of animal crude drugs by the polymerase chain reaction (PCR) technique was attempted for the identification of the biological origin (Hashimoto *et al.*, 1998a; Stanton *et al.*, 1998). This was already applied in a

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variety of research fields in taxonomy, phylogenic analysis, genetics, and disease identification (Carr *et al.*, 1986; Tamate and Tsuchiya, 1995; Comincini *et al.*, 1996; Han *et al.*, 1998; Chung *et al.*, 2000; Lee *et al.*, 2000). Even though this molecular approach does not directly reflect the pharmacological activity, it can be widely used for the confirmation of the presence of animal crude drugs, such as antlers even in the form of extract or powder (Hashimoto *et al.*, 1998b).

In this study, the molecular approach by PCR-RFLP (PCR-restriction fragment length polymorphism) was tried for the discrimination of *Cervidae* (deer) antlers and *Rangifer* (reindeer) antlers, because reindeer antlers are sometimes deceivably displaced as *Cervi Parvum Cornu*.

Materials and Methods

Sample collection Samples of *Cervi Parvum Cornu* imported from China, Russia, Kazakhstan, Hong Kong and New Zealand were collected at the Korea National Quarantine Office, Seoul, Korea. *Cervidae* antlers from red deer, elk and Sika deer were collected from local farms. *Rangifer* antlers from reindeer were purchased from the Kyoungdong Market, Dongdaemun-ku, Seoul. All of the samples were de-haired and then ground to powder.

DNA primers For the amplification of mitochondrial DNA, four different primers were designed and supplied from the Bioneer Co., Cheongwon, Korea. The primer L1091 (5'-AAAAAGCTTCAAAC TGGGATTAGATACCCCACTAT-3'; 35-mer) and H1478 (5'-TGA CTGCAGAGGGTGACGGGCGGTGT GT-3'; 28-mer) were for the amplification of mitochondrial 12S rRNA gene. The primer L14841 (5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGA AA-3'; 35-mer), and H15149 (5'-AAACTGCAGCCCCTCAGAAT GATATTTGTCCTCA-3'; 33-mer) were for the amplification of the cytochrome b gene (Hashimoto *et al.*, 1998a).

DNA amplification Total DNAs were extracted from *Cervi Parvum Cornu* powder and other deer antler powder, by the procedure of Hashimoto *et al.* (1997). The mitochondrial DNA isolated from antlers were amplified by using GeneAmp PCR System 2400 (Perkin Elmer, CT, USA). The reaction mixture (50 μl) was composed of 2 μl of a DNA solution, 5 μl of 10× PCR buffer (100 mM Tris, pH 8.3, 500 mM KCl, 25 mM $MgCl_2$), 4 μl of 2.5 mM dNTP, 2 μl each of 25 μM forward primer and reverse primer, 0.4 μl of 5 unit/mL *Taq* DNA polymerase, and 34.6 μl sterilized water. The amplification cycle was set for 40 times of DNA denaturation at 94°C for 40 s, DNA annealing at 55°C for 1 min, and DNA synthesis at 72°C for 2 min, except for the initial denaturation at 94°C for 5 min.

PCR-RFLP Analysis The PCR products were subjected to restriction digestion by using restriction enzymes including *Taq*I, *Hae*III, *Alu*I, *Hpa*II, and *Sau*3AI. The DNA fragments were separated on 1.7% agarose gel and stained by 0.5 μg/ml ethidium bromide solution. GeneRuler™ 100 bp DNA ladder from MBI Fermentas Inc. was employed as a molecular marker in the agarose gel electrophoresis. The length polymorphism of restriction fragments was analyzed by the comparison of the DNA bands.

DNA Sequencing The amplified PCR products on agarose gel were purified with a JET pure kit (GENOMED Inc., USA). This DNA fragment was inserted into the pGEM-T vector (Promega Corporation, WI, USA) by T4 DNA ligase, and the ligated mixture was used to transform *E. coli* JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)*) (Sambrook *et al.*, 1989). From the white colonies on the LB media (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.2) containing 50 μg/ml ampicillin, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 0.04% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), the plasmid was isolated and subjected to nucleotide sequencing. The sequence determination was performed using T7/SP6 universal primers by the custom service of the Bioneer Co. (Cheongwon, Korea). The determined nucleotide sequence was further analyzed using the GeneCutter Program (http://www.bioneer.co.kr/bioneer_ie.php3).

Results and Discussion

PCR Analysis of Mitochondrial DNAs from *Cervidae* Antlers and *Rangifer* Antlers In order to develop the identification method for the biological origin of *Cervi Parvum Cornu*, PCR-RFLP of mitochondrial DNA was attempted. For this, the 12S rRNA gene and cytochrome b gene in mitochondrial DNA was initially amplified from DNA samples of *Cervidae* and *Rangifer* antlers. There were no differences observed between PCR products of the 12S rRNA gene and cytochrome b gene from the two different species (Fig. 1). All of the samples gave 450 bp bands of the 12S rRNA gene fragments amplified using the L1091 and H1478 primers, and the 380 bp bands of cytochrome b gene fragments amplified using the L14841 and H15149 primer.

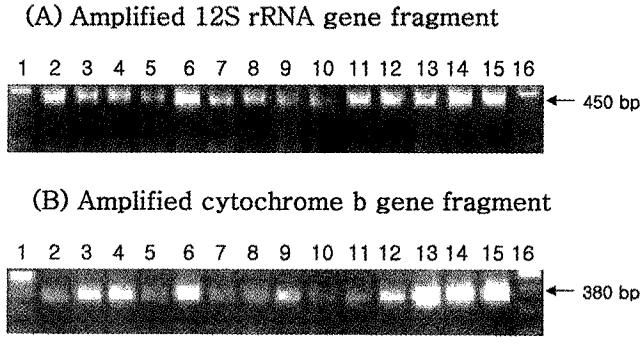

Fig. 1. The amplified mitochondrial DNA fragments from *Cervidae* and *Rangifer* antlers. (A) The 12S rRNA gene fragment amplified by PCR using L1091 and H1478 primers. (B) The cytochrome b gene fragment amplified by PCR using L14841 and H15149 primers. Lanes 1 and 16, GeneRuler™ 100 bp DNA ladder; lane 2, antler of Chinese deer; lane 3, antler of Russian deer; lane 4, antler of New Zealand deer; lane 5, antler of Hong Kong deer; lane 6, antler of Kazakhstan deer; lanes 7 and 8, antlers of red deer; lanes 9 and 10, antlers of elk; lanes 11 and 12, antlers of Sika deer; and lanes 13, 14 and 15, antlers of 3 different reindeer sources.

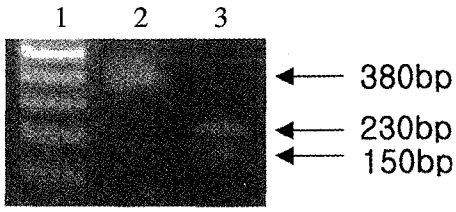

Fig. 2. The restriction digestion pattern of the amplified mitochondrial cytochrome b gene fragments by *Taq*I enzyme. The mitochondrial cytochrome b gene fragments amplified by PCR using L14841 and H15149 primers were subjected to restriction digestion with *Taq*I. Lane 1, GeneRuler™ 100 bp DNA ladder; lane 2, *Cervi Parvum Cornu* powder; lane 3, antler powder of reindeer.

For a precise analysis, all of the amplified PCR products were subjected to a RFLP analysis using *Taq*I, a 4-bp recognition enzyme. Particularly, the PCR products of the cytochrome b gene that was amplified using the L14841 and H15149 primer (380 bp) showed different restriction patterns between the *Cervidae* and *Rangifer* antlers (Fig. 2). However, no difference was observed in the restriction patterns of the amplified mitochondrial 12S rRNA gene with some endonucleases (data not shown). The typical discriminative PCR-RFLP pattern of the *Cervidae* antler with *Taq*I was observed as an undigested 380 bp fragment, which is different from the 230 bp and 150 bp bands from the *Rangifer* antler. The result suggests that the PCR-RFLP analysis is a powerful tool in identifying the biological origin of the *Cervidae* antlers from the *Rangifer* antlers.

Sequence Determination and Restriction Analysis of Mitochondrial Cytochrome b Gene Fragments In order to confirm the PCR-RFLP data in Fig. 2, the base sequences of the amplified PCR products from the *Cervidae* antlers and the *Rangifer* antlers were determined (Fig. 3). The sequence comparison showed a 85.1% homology between the two amplified mitochondrial cytochrome b gene fragments from the *Cervidae* and *Rangifer* antlers, excluding the primer parts at both ends (262 bp among 308 bp).

The restriction analysis of the determined sequences of the mitochondrial cytochrome b region also showed that the *Rangifer* species could be simply discriminated from other *Cervidae* antlers by a PCR-RFLP analysis, using *Alu*I, *Hae*III, *Hpa*II or *Sau*3AI(*Mbo*I), as well as *Taq*I (Table 1).

Molecular Discrimination of the Mitochondrial Cytochrome b Gene from *Cervidae* and *Rangifer* Antlers Based on the restriction analysis of the amplified sequences, a PCR-RFLP analysis was conducted in the region of the mitochondrial cytochrome b gene from 8 *Cervidae* (Chinese deer, Russian deer, Hong Kong deer, New Zealand deer, Kazakhstan deer, red deer, elk and Sika deer) antlers and 3 *Rangifer* (reindeer) antlers (Fig. 4.) Especially when PCR products of the mitochondrial cytochrome b gene fragment were cut by *Alu*I or *Hpa*II, *Cervidae* antlers gave only one

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          AluI                                                                70
Deer  AAAAAGCTTC CATCCAACAT CTCAGCATGA TGAAACTTAG GCTCCCTGCT AGGAATCTGC TTAATCCTAC
Rein  ********** ********** ********** *****T**T* ****T**A** ********** *****T****
      AAAAAGCTTC CATCCAACAT CTCAGCATGA TGAAA (L14841)

          HpaII/HaeIII                                                      140
Deer  AAATCCTAAC AGGCCTATTC CTATCAATAC ACTATACATC CGACACAATA ACAGTATTCT ACTCCGTTAC
Rein  *******T** C**T*****T ***G****** *T******** ***T****** ****C***** C***T*****

           TaqI                                                             210
Deer  CCATATTTGC CGAGATATCA ATTATGGCTG AATTATCCGA TACATACATG CAAACGGAGC AATAATATTC
Rein  T**C**C**T *****CG*** ********** ***C****** **T******* *C******** *TC******T

                                    HaeIII       Sau3AI                     280
Deer  TTCATTTGCC TATTTATACA TGTAGGACAA GGCCTATACT ATGGGTTATA TGCTTTCCTA GAAACATGAA
Rein  *****C***T ********** ********G* ********** *C**A*C*** CA*C****** **********

              Sau3AI                 AluI                                   350
Deer  ACATCGGAGT AATTCTTCTA TTCACAGTGA CAGCCACAGC ATTTGTAGGA TATGTCCTAC CATGAGGACA
Rein  *T**T***** G**C**CT** **T*****A* T***T***** ********** ********** **********
                                                                  TGAGGACA

                                376
Deer  AATATCATTC TGAGGGGCTG CAGTTT
Rein  ********** ********** ******
      AATATCATTC TGAGGGGCTG CAGTTT (H15149)
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Fig. 3. The nucleotide sequences of the mitochondrial cytochrome b gene fragments amplified from *Cervidae* and *Rangifer* antlers. The nucleotide sequences of mitochondrial cytochrome b gene fragments amplified by PCR using L14841 and H15149 primers were determined by a dideoxy chain termination reaction using T7/SP6 universal primers. Deer, nucleotide sequence of the mitochondrial cytochrome b gene fragment from *Cervidae* (deer) antler; Rein, from *Rangifer* (reindeer) antler. The primers of L14841 and H15149 were underlined at both ends, and the restriction sites of *Alu*I, *Hae*III, *Hpa*II, *Sau*3AI and *Taq*I were marked in gray letters.

Table 1. Restriction analysis of amplified mitochondrial cytochrome b gene fragments based on the determined base sequences.

Primers		L14841 + H15149 (Cytochrome b gene)	
Antler Source		*Cervidae* sp.	*Rangifer* sp.
PCR Product (bp)		376	376
PCR-RFLP Product (bp)	*Alu*I	376	307 + 63
	*Hae*III	159 + 134 + 83	242 + 134
	*Hpa*II	376	296 + 80
	*Sau*3A	376	253 + 86 + 37
	*Taq*I	376	226 + 150

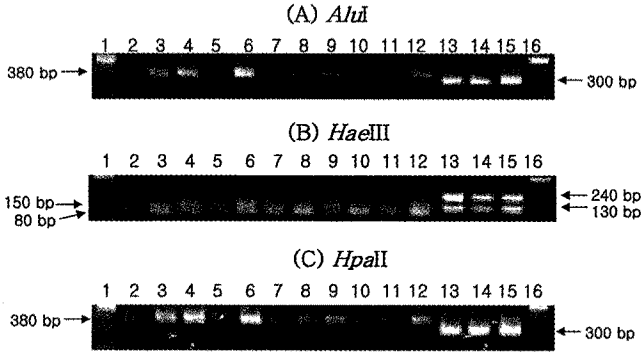

Fig. 4. The PCR-RFLP analysis pattern of the mitochondrial cytochrome b gene fragments amplified from *Cervidae* and *Rangifer* antlers. The mitochondrial cytochrome b gene fragments amplified from *Cervidae* and *Rangifer* antlers were digested with 4-bp recognition enzymes, *Alu*I (A), *Hae*III (B) and *Hpa*II (C). Lanes 1 and 16, GeneRuler™ 100 bp DNA ladder; lane 2, antler of Chinese deer; lane 3, antler of Russian deer; lane 4, antler of New Zealand deer; lane 5, antler of Hong Kong deer; lane 6, antler of Kazakhstan deer; lanes 7 and 8, antlers of red deer; lanes 9 and 10, antlers of elk; lane 11s and 12, antlers of Sika deer; and lanes 13, 14 and 15, antlers of 3 different reindeer sources.

DNA band at the undigested state, but a shorter DNA band appeared in the amplified PCR products from *Rangifer* antlers. A PCR-RFLP analysis using *Hae*III showed two distinctive DNA bands of 150 bp and 80 bp in *Cervidae* samples, compared to 240 bp and 130 bp in the *Rangifer* samples.

However, this molecular identification procedure is still inadequate since it does not directly reflect the pharmacological activity of antlers from different species or different regions. Nevertheless, it will be a more convenient method in detecting the biological source or origin of antlers, even in a powder state or extract in mixed drug formulas (Hashimoto *et al.*, 1998b), since it needs only a small sample and it provides rapid identification.

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