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Cervus 종의 Phylogenetic analysis에 의한 판별

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Authentication of *Cervus* Species by Phylogenetic analysis

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ABSTRACT

Objectives : This study was performed to determine if an antler could be identified as one of the *Cervus* species by phylogenetic analysis, which was used to assess genetic authentication.

Methods : The DNAs of an antler were extracted, amplified by PCR, and sequenced. The DNAs of an antler were identified by Phylogenetic analysis. Phylogenetic analysis was made using MEGA software (Molecular Evolutionary Genetics Analysis, 3.1).

Results : By phylogenetic analysis an antler was identified as *Cervus elaphus nelsoni* not as *Cervus elaphus sibericus*. This work showed that authentication can efficiently be performed by phylogenetic analysis.

Conclusion : These results suggest that phylogenetic analysis might be able to provide the authentication of *Cervus* species.

Key words : genetic authentication, *Cervus* species, MEGA

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Introduction

Some deer horns have been used as traditional Korean medicines for hundreds of years[1]. But it is undesirable that different *Cervus* species might be used under the same name. For example, some *Cervus elaphus nelsoni* in Canada having Chronic Wasting Disease (CWD) since December, 2000. The horns of these *Cervus* species are very similar in appearance when they were sliced especially. The methods of identification among *Cervus* species, however, have not been recorded in traditional Korean or Chinese herbal literatures.

Characterization and scoring of genetic variations are increasingly important to correlate phenotypical and genotypical differences. We investigated the possibility to determine *Cervus* species in DNA by using phylogenetic analysis, which was used to assess genetic identification.

Genetic identification of traditional Korean herbs should help to ensure the safe use of traditional Korean herbal materials. So, the method for identifying the origin is very important.

Until now there have been several genetic studies on *Cervus* species. But genetic authentication of *Cervus* species is rare. This study was performed to determine if *Cervus* species could be identified by phylogenetic analysis, which was used to assess genetic identification.

Materials and Methods

1. Samples and Purification of DNA

An antler of *Cervus* species were sent to the Research Center for Biomedical Resources of Oriental Medicine, Daegu Haany University (Korea) by an Oriental Medical doctor in September, 2005. We named this samples of antler as NY-C. Three g of NY-C in the form of slice was firstly minced with a sterilized scalpel and pulverized to powder by using a sterilized mortar and pestle. A DNA isolation kit (DNeasy, No. 69504) (Qiagen Inc., Valencia, CA) was used as described in the manufacturer's instructions with slight modifications. 300 mg of the powdered sample was used in the purification procedure. Before sample elution, the columns were dried at 37℃ for 5 minutes to evaporate residual ethanol. Samples were eluted in a total volume of 200 μl of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)].

2. Preparation for sequencing

The extracted DNA was amplified by polymerase chain reaction (PCR). A 1200-bp region of mitochondrial D-loop of NY-C was amplified using 25 ng of DNA, 5 pmol of each primer; forward was 5′-TAATATACTGGTCTTGTAAACC -3′ and reverse was 5′- GGGTCGGAAGGCTGGGACCAAACC -3′. The PCR amplification was performed by using 0.5 unit Taq polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom). The 30 μl of PCR reaction mixtures were 10 mM Tris-HCl, pH 9.0, 1.5 mM magnesium chloride, 50 mM potassium chloride, 0.1% Triton-X 100, 0.01% [v/v] stabilizer, 0.25 mM of each deoxynucleotide triphosphate (dNTP), 0.1 M of each oligonucleotide primer. The PCR steps were denaturation of 5 minutes at 95℃, 30 cycles of 30 seconds at 95℃, 30 seconds at 60℃, and 30 seconds at 72℃ with a PCR System (Astec, Fukuoka, Japan). The quality of PCR products was controlled by 1.5% of agarose gel electrophoresis.

3. Sequencing

All amplicons were purified using the PCR-M Clean Up System (Viogene). The DNA fragments were sequenced using an ABI Prism 377 automated DNA sequencer (Applied Biosystems) with a BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems). All amplicons were sequenced on both strands using primers of D-loop. We sequenced at six times to make sure there were no errors in the results.

4. Phylogenetic analysis

Phylogenetic analysis was made using MEGA software (Molecular Evolutionary Genetics Analysis, 3.1)[2]. MEGA software is a package of applications

for molecular genetics analysis. The sequences were compared to reference data available at the GenBank database by using Basic Local Alignment Search Tool (BLAST)[3]. A dendrogram was formed with the Kimura 2-parameter distance method and bootstrap was tested with 1000 replications.

Results

1. Sequencing Data

We sequenced at six times and named as 5D_NY-C-1200S, A3_NY-C-F1, A4_NY-C-F1_05, C5_NY-C-NY1306-S_06, C6_NY-C-NY1306-S_01, and D6_NY-C-1200S_06. The sequences obtained in our study were shown in **Figure 1**.

>5D_NY-C-1200S

GAAGAAGCCATAGCCCCACTATCAACACCCAAAGCTGAAGTTCTAT
TTAAACTATTCCCTGACGCTTATTAATATAGTTCCATAAAAATCAAGA
ACTTTATCAGTATTAAATTTCCAAAAAATTTAATATTTTAATACAGC
TTTCTACTCNACATCCAATTTACATTTTATGTCCTACTAATTACACAG
CAAAACACGTGATATAACCTTATGCGCTCGTAGTACATAAAATCAATG
GGCTAGGACATGCATGTATAACAGTACATGAGTTAGCGTATAGGACAT
ATTATGTATAATAGTACATAAATTAATGTATTAAGACATACTATGTA
TAATAGTACATTATATTATATGCCCCATGCTTATAAGCATGGACTTCT
CATCATTTAAAGTACATAGTACATAATGTTGTTCATCGTACATAGCAC
ATTAAGTCAAATCAGTCCTTGTCAACATGCGTATCCCGCCCCCTAGATC
ACGAGCTTAATTACCATGCCGCGTGAAACCAGCAACCCGCTGGGCAGGG
ATCCCTCTTCTCGCTCCGGGCCCATGAACCGTGGGGGTAGCTATTTAAT
GAACTTT

>A3_NY-C-F1

ACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGACGCTTATT
AATATAGTTCCATAAAAATCAAGAACTTTATCAGTATTAAATTTCCA
AAAAATTTAATATTTTAATACAGCTTTCTACTCAACATCCAATTTACA
TTTTATGTCCTACTAATTACACAGCAAAACACGTGATATAACCTTATG
CGCTCGTAGTACATAAAATCAATGTGCTAGGACATGCATGTATAACAG
TACATGAGTTAGCGTATAGGACATATTATGTATAATAGTACATAAAT
TAATGTATTAAGACATACTATGTATAATAGTACATTATATTATATGC
CCCATGCTTATAAGCATGTACTTCTCATCATTTAAAGTACATAGTACA
TAATGTTGTTCATCGTACATAGCACATTAAGTCAAATCAGTCCTTGTC
AACATGCGTATCCCGCCCCCTAGATCACGAGCTTAATTACCATGCCGCG
TGAAACCAGCAACCCGCTGGGCAGGGATCCCTCTTCTCGCTCCGGGCCCA
TGAACCGTGGGGGTAGCTATTTAATGAACTTTATCAGACATCTGGTTC
TTTTTTCAGGCATCTCATCT

>A4_NY-C-F1_05

AGCTGAAGTTCTATTTAAACTATTCCCTGACGCTTATTAATATAGT
TCCATAAAAATCAAGAACTTTATCAGTATTAAATTTCCAAAAAATTT
AATATTTTAATACAGCTTTCTACTCAACATCCAATTTACATTTTATGT
CCTACTAATTACACAGCAAAACACGTGATATAACCTTATGCGCTCGTA
GTACATAAAATCAATGTGCTAGGACATGCATGTATAACAGTACATGAG
TTAGCGTATAGGACATATTATGTATAATAGTACATAAATTAATGTAT
TAAGACATACTATGTATAATAGTACATTATATTATATGCCCCATGCTT
ATAAGCATGTACTTCTCATCATTTAAAGTACATAGTACATAATGTTGT
TCATCGTACATAGCACATTAAGTCAAATCAGTCCTTGTCAACATGCGT
ATCCCGCCCCCTAGATCACGAGCTTAATTACCATGCCGCGTGAAACCAG
CAACCCGCTGGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATGAACCGTG
GGGGTAGCTATTTAATGAACTTTATCAGACATCTGGTTCTTTTTTCAG
GCATCTC

>C5_NY-C-NY1306-S_06

TATCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGACGC
TTATTAATATAGTTCCATAAAAATCAAGAACTTTATCAGTATTAAAT
TTCCAAAAAATTTAATATTTTAATACAGCTTTCTACTCAACATCCAAT
TTACATTTTATGTCCTACTAATTACACAGCAAAACACGTGATATAACC
TTATGCGCTCGTAGTACATAAAATCAATGTGCTAGGACATGCATGTAT
AACAGTACATGAGTTAGCGTATAGGACATATTATGTATAATAGTACAT
AAATTAATGTATTAAGACATACTATGTATAATAGTACATTATATTAT
ATGCCCCATGCTTATAAGCATGTACTTCTCATCATTTAAAGTACATAG
TACATAATGTTGTTCATCGTACATAGCACATTAAGTCAAATCAGTCCT
TGTCAACATGCGTATCCCGCCCCCTAGATCACGAGCTTAATTACCATGC
CGCGTGAAACCAGCAACCCGCTGGGCAGGGATCCCTCTTCTCGCTCCGGG
CCCATGAACCGTGGGGGTAGCTATTTAATGAACTTTATCAGACATCTG

>C6_NY-C-NY1306-S_01

TATCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGACGC
TTATTAATATAGTTCCATAAAAATCAAGAACTTTATCAGTATTAAAT
TTCCAAAAAATTTAATATTTTAATACAGCTTTCTACTCAACATCCAAT
TTACATTTTATGTCCTACTAATTACACAGCAAAACACGTGATATAACC
TTATGCGCTCGTAGTACATAAAATCAATGTGCTAGGACATGCATGTAT
AACAGTACATGAGTTAGCGTATAGGACATATTATGTATAATAGTACAT
AAATTAATGTATTAAGACATACTATGTATAATAGTACATTATATTAT
ATGCCCCATGCTTATAAGCATGTACTTCTCATCATTTAAAGTACATAG
TACATAATGTTGTTCATCGTACATAGCACATTAAGTCAAATCAGTCCT
TGTCAACATGCGTATCCCGCCCCCTAGATCACGAGCTTAATTACCATGC
CGCGTGAAACCAGCAACCCGCTGGGCAGGGATCCCTCTTCTCGCTCCGGG
CCCATGAACCGTGGGGGTAGCTATTTAATGAACTTTATCAGACATCTG

GTTCTTTTTTCAGGGCCATCTCATCTAAAATCGCCCACTCCTTGTAATA
TAAGACATCTCGATGGACTAATGACTAATCAGCCCATGCTCACACATA
ACTGTGGTGTCATACATTTGGTATTTTTAATTTTTGGGGGGATGCTTG
GGACTCAGCA

>D6_NY-C-1200S_06

AAGAAGCCATAGCCCCACTATCAACACCCAAAGCTGAAGTTCTATT
TAAACTATTCCCTGACGCTTATTAATATAGTTCCATAAAAATCAAGAA
CTTTATCAGTATTAAATTTCCAAAAAATTTAATATTTTAATACAGCT
TTCTACTCNACATCCAATTTACATTTTATGTCCTACTAATTACACAGC
AAAACACGTGATATAACCTTATGCGCTCGTAGTACATAAAATCAATGG
GCTAGGACATGCATGTATAACAGTACATGAGTTAGCGTATAGGACATA
TTATGTATAATAGTACATAAATTAATGTATTAAGACATACTATGTAT
AATAGTACATTATATTATATGCCCCATGCTTATAAGCATGTACTTCTC
ATCATTTAAAGTACATAGTACATAATGTTGTTCATCGTACATAGCACA
TTAAGTCAAATCAGTCCTTGTCAACATGCGTATCCCGCCCCCTAGATCA
CGAGCTTAATTACCATGCCGCGTGAAACCAGCAACCCGCTGGGCAGGGA
TCCCTCTTCTCGCTCCGGGCCCATGAACCGTGGGGGTAGCTATTTAATG
AACTTTATCAGACATCTGGTTCTTTTTTCAGGGCCATCTCATCTAAAA
TCGCCCACTCCGTTGG

Figure 1. Sequences of an antler (NY-C). We sequenced at six times and named as 5D_NY-C-1200S, A3_NY-C-F1, A4_NY-C-F1_05, C5_NY-C-NY1306-S_06, C6_NY-C-NY1306-S_01, and D6_NY-C-1200S_06.

2. Genetic authentication

By phylogenetic analysis all sequences (5D_NY-C-1200S, A3_NY-C-F1, A4_NY-C-F1_05, C5_NY-C-NY1306-S_06, C6_NY-C-NY1306-S_01, and D6_NY-C-1200S_06.) of an antler NY-C were more related to Cervus elaphus nelsoni than Cervus elaphus sibericus and so on. Phylogenetic gene tree was shown in **Figure 2**.

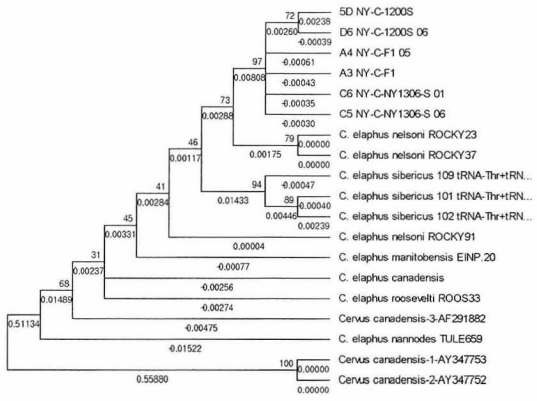

Figure 2. Gene tree results of antler (NY-C). NY-C was close to Cervus elaphus nelsoni. Unrooted tree shows Neighbor-Joining analysis based on D-loop segment sequences (Kimura 2-parameter distance, bootstrap test with 1000 replications). 5D_NY-C-1200S, A3_NY-C-F1, A4_NY-C-F1_05, C5_NY-C-NY1306-S_06, C6_NY-C-NY1306-S_01, and D6_NY-C-1200S_06 were sequences of an antler (NY-C).

Discussion

Cervus species have been used as an important medicine in traditional Korean medicines for a long time. They have been widely distributed in the world including East Asia. Their horns have been used as tonic genera in general.

It is undesirable when different *Cervus* species might be used under the same name, especially it is very harmful in case of CWD. And many commercial *Cervus* species products are extremely difficult to identify in the form of powder, or extracts. The authentication via analysing chemical profiles is also very difficult for many variables such as the analysing condition and nutritional factors.

From phylogenetic analysis one of *Cervus* species was identified as not *Cervus elaphus sibericus* but *Cervus elaphus nelsoni*. This works showed that identification can efficiently be performed by phylogenetic analysis. These results suggest that sequencing and phylogenetic analysis methods are suitable for authentication of the concerned *Cervus* species.

A few dealers practiced a deception of disguising one antler from a certain country as other country. Because a general consumer could not distinguish the provenance of antlers. But in our study we couldn't identify reared places or areas of *Cervus elaphus* species.

Results of this study leaved more to be investigated and answered, but they proposed the useful tool for identification of *Cervus* species. This is useful in determining the relatedness of our organisms to the one in the database.

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