대한한의학회지 제27권 제2호(2006년 6월)
J Korean Oriental Med 2006;27(2):225-231

원 저

Genetic Distance Methods for the Identification of *Cervus* Species

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Objectives : This study was performed to determine if unknown species of antler samples could be identified by genetic distance methods.

Methods : The DNAs of 4 antler samples were extracted, amplified by PCR, and sequenced. The DNAs of antlers were identified by genetic distance. Genetic distance method was made using MEGA software (Molecular Evolutionary Genetics Analysis, 3.1).

Results : By genetic distance methods, all 4 antler samples were closest to *Cervus elaphus nelsoni* among *Cervus* species.

Conclusion : These results suggest that genetic distance methods might be used as a tool for the identification of *Cervus* species.

Key Words: Genetic distance, identification, *Cervus* species.

Introduction

Deer have been widely distributed around the world, including East Asia. Their horns have been used as tonic genera in general in traditional Korean medicines for a long time.[1)] There are many species of deer in the world, and it is undesirable that different *Cervus* species be used under the same name. For example, some *Cervus elaphus nelsoni* in Canada have contracted bovine spongiform encephalopathy (BSE) since December 2000. The horns of these *Cervus* species are very similar in appearance. Also, many commercial *Cervus* species products are extremely difficult to identify in the form of slices, powder, or extracts, so characterization and scoring of genetic variations are increasingly important to correlate phenotypical and genotypical differences. The measure of the genetic distance between organisms is one of the most important tasks in molecular evolution.

· 접수 : 2006년 5월 22일 · 논문심사 : 2006년 5월 23일
· 채택 : 2006년 6월 10일

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· This work was supported by grant number (R12-2003-002-00000-0) from the basic research program of the Ministry of Commerce, Industry and Energy, Republic of Korea.

Genetic distance is usually obtained by computing the number of nucleotide or amino acid substitutions between sequences.

This study was performed to determine if unknown species of antler samples could be identified by genetic distance methods.

Materials and Methods

1. Samples and Purification of DNA

Four unknown species of antler samples were sent to the Research Center for Biomedical Resources of Oriental Medicine, Daegu Hanny University (Korea) by an Oriental medical doctor in September 2005. We labeled these samples of antler NY-B, NY-C, NY-D and NY-E. Three g of antler samples in the form of slice were first 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, USA) 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 DNAs were amplified by polymerase chain reaction (PCR). A 1200-bp region of mitochondrial D-loop of antlers were amplified using 25 ng of DNA, 5 pmol of each primer; forward was 5'- TAATATACTGGTCTTGTAAACC -3' and reverse was 5'- GGGTCGGAAGGCTGGGACCAAACC

Table 1. Pairwise distances of the nucleotide sequences of NY-B based on D-loop segment sequences.

[	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
[1]															
[2]	0.008														
[3]	0.039	0.048													
[4]	0.008	0.006	0.045												
[5]	0.011	0.010	0.041	0.006											
[6]	0.013	0.008	0.046	0.008	0.011										
[7]	0.000	0.008	0.039	0.008	0.011	0.013									
[8]	0.005	0.003	0.045	0.003	0.006	0.008	0.005								
[9]	0.024	0.023	0.045	0.023	0.019	0.024	0.024	0.019							
[10]	0.033	0.031	0.053	0.031	0.028	0.031	0.033	0.028	0.008						
[11]	0.029	0028	0.050	0.028	0.024	0.028	0.029	0.024	0.005	0.006					
[12]	0.038	0.046	0.002	0.043	0.039	0.045	0.038	0.043	0.043	0.051	0.048				
[13]	0.008	0.003	0.045	0.003	0.006	0.005	0.008	0.003	0.023	0.031	0.028	0.043			
[14]	1.073	1.062	1.079	1.056	1.073	1.031	1.073	1.073	1.081	1.094	1.094	1.072	1.056		
[15]	1.073	1.062	1.079	1.056	1.073	1.031	1.073	1.073	1.081	1.094	1.094	1.072	1.056	0.000	

The distances were calculated using MEGA (Kimura 2-parameter model) (Kumar et al., 2004). [1] C._elaphus_nelsoni_ROCKY23, [2] C._elaphus_manitobensis_EINP.20, [3] NY-B-total, [4] C._elaphus_roosevelti_ROOS33, [5] C._elaphus_canadensis, [6] C._elaphus_nannodes_TULE659, [7] C._elaphus_nelsoni_ROCKY37, [8] C._elaphus_nelsoni_ROCKY91, [9] C._elaphus_sibericus_109_tRNA-Thr+tRN, [10] C._elaphus_sibericus_101_tRNA-Thr+tRN, [11] C._elaphus_sibericus_102_tRNA-Thr+tRN, [12] B5_NY-B-NY1306-S, [13] Cervus_canadensis-3-AF291882, [14] Cervus_canadensis-1-AY347753, [15] Cervus_canadensis-2-AY347752

-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 them six times to make sure there were no errors in the results. The sequences were proofread and aligned and consensus sequences (NY-B-total, NY-C-total, NY-D-total and NY-E-total) were obtained with SeqMan II (DNASTAR).

4. Genetic distance

Genetic distance was established 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]. The distances were calculated

Table 2. Pairwise distances of the nucleotide sequences of NY-C based on D-loop segment sequences.

[	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
[1]															
[2]	0.011														
[3]	0.008	0.009													
[4]	0.011	0.012	0.006												
[5]	0.008	0.015	0.012	0.015											
[6]	0.008	0.0185	0.012	0.015	0.000										
[7]	0.012	0.011	0.008	0.011	0.014	0.014									
[8]	0.000	0.011	0.008	0.011	0.008	0.008	0.012								
[9]	0.005	0.006	0.003	0.006	0.009	0.009	0.008	0.005							
[10]	0.022	0.023	0.020	0.017	0.023	0.023	0.022	0.022	0.017						
[11]	0.030	0.031	0.028	0.025	0.031	0.031	0.028	0.030	0.025	0.008					
[12]	0.027	0.028	0.025	0.022	0.028	0.028	0.025	0.027	0.022	0.005	0.006				
[13]	0.008	0.006	0.003	0.006	0.12	0.012	0.005	0.008	0.003	0.020	0.028	0.025			
[14]	1.065	1.055	1.049	1.049	1.057	1.057	1.025	1.065	1.065	1.041	1.037	1.037	1.049		
[15]	1.059	1.049	1.043	1.043	1.051	1.051	1.020	1.059	1.059	1.035	1.031	1.031	1.043	0.002	

The distances were calculated using MEGA (Kimura 2-parameter model) (Kumar et al., 2004). [1] C._elaphus_nelsoni_ROCKY23, [2] C._elaphus_manitobensis_EINP.20, [3] C._elaphus_roosevelti_ROOS33, [4] C._elaphus_canadensis, [5] NY-C-total, [6] C6_NY-C-NY1306-S_01, [7] C._elaphus_nannodes_TULE659, [8] C._elaphus_nelsoni_ROCKY37, [9] C._elaphus_nelsoni_ROCKY91, [10] C._elaphus_sibericus_109_tRNA-Thr+tRN, [11] C._elaphus_sibericus_101_tRNA-Thr+tRN, [12] C._elaphus_sibericus_102_tRNA-Thr+tRN, [13] Cervus_canadensis-3-AF291882, [14] Cervus_canadensis-1-AY347753, [15] Cervus_canadensis-2-AY347752

using Kimura 2-parameter model[4].

Results

1. Sequencing Data

We sequenced at six times and selected 2 sequences from each 4 antler samples for analyses: B5_NY-B-NY1306-S and NY-B-total from NY-B, C6_NY-C-NY1306-S_01 and NY-C-total from NY-C, D6_NY-D-NY1306-S and NY-D-total from NY-D, E5_NY-E-NY1306-S and NY-E-total from NY-E. The sequences obtained in our study are shown in Figure 1.

2. Pairwise distance calculation

Distance matrices from aligned nucleotide sequences were determined by using the pairwise distance calculation of MEGA applying the Kimura 2-parameter model. The pairwise genetic distances obtained in our study were shown in Table 1-4. From genetic distances methods 4 antler samples, NY-B, NY-C, NY-D and NY-E, were all closest to Cervus elaphus nelsoni among *Cervus* species (C._elaphus_nelsoni_ROCKY23, C._elaphus_manitobensis_EINP.20, C._elaphus_roosevelti_ROOS33, C._elaphus_canadensis, C._elaphus_nannodes_TULE659, C._elaphus_nelsoni_ROCKY37, C._elaphus_nelsoni_ROCKY91, C._elaphus_sibericus_109_tRNA-Thr+tRN, C._elaphus_sibericus_101_tRNA-Thr+tRN, C._elaphus_sibericus_102_tRNA-Thr+tRN, Cervus_canadensis-3-AF291882, Cervus_canadensis-1-AY347753, Cervus_canadensis-2-AY347752).

Discussion

Some deer horns have been used as traditional Korean medicines for hundreds of years [1]. It is

Table 3. Pairwise distances of the nucleotide sequences of NY-D based on D-loop segment sequences.

[	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
[1]															
[2]	0.009														
[3]	0.009	0.007													
[4]	0.012	0.010	0.007												
[5]	0.014	0.009	0.009	0.012											
[6]	0.000	0.009	0.009	0.012	0.014										
[7]	0.023	0.028	0.028	0.024	0.030	0.023									
[8]	0.015	0.017	0.017	0.017	0.019	0.015	0.021								
[9]	0.005	0.003	0.003	0.007	0.009	0.005	0.024	0.014							
[10]	0.023	0.021	0.021	0.017	0.023	0.023	0.028	0.021	0.017						
[11]	0.031	0.030	0.030	0.026	0.030	0.031	0.037	0.030	0.026	0.009					
[12]	0.028	0.026	0.026	0.023	0.026	0.028	0.033	0.026	0.023	0.005	0.007				
[13]	0.009	0.003	0.003	0.007	0.005	0.009	0.028	0.017	0.003	0.021	0.030	0.026			
[14]	1.064	1.053	1.047	1.047	1.021	1.064	1.021	1.038	1.064	1.029	1.025	1.025	1.047		
[15]	1.064	1.053	1.047	1.047	1.021	1.064	1.021	1.038	1.064	1.029	1.025	1.025	1.047	0.000	

The distances were calculated using MEGA (Kimura 2-parameter model) (Kumar et al., 2004). [1] C._elaphus_nelsoni_ROCKY23, [2] C._elaphus_manitobensis_EINP.20, [3] C._elaphus_roosevelti_ROOS33, [4] C._elaphus_canadensis, [5] C._elaphus_nannodes_TULE659, [6] C._elaphus_nelsoni_ROCKY37, [7] NY-D-total, [8] D6_NY-D-NY1306-S, [9] C._elaphus_nelsoni_ROCKY91, [10] C._elaphus_sibericus_109_tRNA-Thr+tRN, [11] C._elaphus_sibericus_101_tRNA-Thr+tRN, [12] C._elaphus_sibericus_102_tRNA-Thr+tRN, [13] Cervus_canadensis-3-AF291882, [14] Cervus_canadensis-1-AY347753, [15] Cervus_canadensis-2-AY347752

undesirable that different *Cervus* species might be used under the same name, especially as it is potentially very harmful in the case of BSE. Many commercial *Cervus* species products are extremely difficult to identify in the form of powder or extracts. A few dealers practiced deception by disguising antler from a certain country as being from another country because a general consumer could not distinguish the provenance of antlers. The methods of identification among *Cervus* species, however, have not been recorded in traditional Korean or Chinese herbal literatures. The authentication via analyzing chemical profiles is also very difficult due to many variables such as the analyzing condition and nutritional factors.

We investigated the possibility of determining *Cervus* species from DNA by using pairwise genetic distances methods, which was used to assess genetic identification [4,5]. So far, some genetic methods for identification of deer or antlers have been tried, but they were for identification between different species [6,7]. Moreover, in those cases, the genetic distance method was not used.

The measure of the genetic distance between organisms is one of the most important tasks in molecular evolution. Genetic distance is usually obtained by computing the number of nucleotide or amino acid substitutions between sequences.

In this study we estimated genetic distances between a set of DNA sequences. The Kimura 2-parameter nucleotide substitution model was chosen since it is available in most phylogenetic software packages, e.g. Phylip, MEGA and PAUP.

From genetic distances methods 4 antler samples were all close to Cervus elaphus nelsoni.

Table 4. Pairwise distances of the nucleotide sequences of NY-E based on D-loop segment sequences.

[	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
[1]															
[2]	0.008														
[3]	0.008	0.007													
[4]	0.012	0.010	0.007												
[5]	0.013	0.008	0.008	0.012											
[6]	0.000	0.008	0.008	0.012	0.013										
[7]	0.005	0.003	0.003	0.007	0.008	0.005									
[8]	0.022	0.020	0.020	0.017	0.022	0.022	0.017								
[9]	0.018	0.020	0.020	0.020	0.022	0.018	0.017	0.024							
[10]	0.0418	0.020	0.020	0.020	0.022	0.018	0.017	0.024	0.000						
[11]	0.030	0.029	0.029	0.025	0.029	0.030	0.025	0.008	0.032	0.032					
[12]	0.027	0.025	0.025	0.022	0.025	0.027	0.022	0.005	0.029	0.029	0.007				
[13]	0.008	0.003	0.003	0.007	0.005	0.008	0.003	0.020	0.020	0.020	0.029	0.025			
[14]	1.033	1.023	1.017	1.017	0.994	1.033	1.033	1.009	1.023	1.023	1.005	1.005	1.017		
[15]	1.033	1.023	1.017	1.017	0.994	1.033	1.033	1.009	1.023	1.023	1.005	1.005	1.017	0.000	

The distances were calculated using MEGA (Kimura 2-parameter model) (Kumar et al., 2004). [1] C._elaphus_nelsoni_ROCKY23, [2] C._elaphus_manitobensis_EINP.20, [3] C._elaphus_roosevelti_ROOS33, [4] C._elaphus_canadensis, [5] C._elaphus_nannodes_TULE659, [6] C._elaphus_nelsoni_ROCKY37, [7] C._elaphus_nelsoni_ROCKY91, [8] C._elaphus_sibericus_109_tRNA-Thr+tRN, [9] NY-E-total, [10] E5_NY-E-NY1306-S, [11] C._elaphus_sibericus_101_tRNA-Thr+tRN, [12] C._elaphus_sibericus_102_tRNA-Thr+tRN, [13] Cervus _canadensis-3 AF291882, [14] Cervus_canadensis-1 AY347753, [15] Cervus_canadensis-2 AY347752

>B5_NY-B-NY1306-S
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TCAACATCCAATTTACATTTTACCTCCTACTAATTACACAGCAAAACACGTGATATAACCTTATGCGCTTGGAGTACATAAAATCAATGTGCTAGAACACGCATGTATAACAGCACATGAGTTAGTGGA
TAGGACATATTATGTATAATAGTACATAAATCAATGTATTAGGACATATTATGTATAATAGTACATTATATTATATGCCCCATGCTTATAAGCATGGACTTTTCATTATTCAAAGTACATAGTACATA
ATGTTGTTCATCGTACATAGTGCATTAAGTCAAATCAGTCCCTGGCAACATGCGTATCCCGTCCCCTAGATCACGAGCTTAATTACCATGCCGCGGGAAACCAGCAACCCGCTGGGCAGGGATCCCTCTTC
TCGCTCCGGGCCCATAAACCGTGGGGGTAGCTATTTAATGAACTTTATCAGACATCTGGTTCTTTTTTCAGGGCCATCTCACCTAAAATCGCCCACTCCTTGCAATATAAGACATCTCGATGGACTAATGA
CTAATCAGCCCATGCTCACACATAACTGTGGTGGCATACATTTGGGATTTTTAA
>NY-B-total
TAGgTSTGGnTTTGGTCCCAGCCTTCCGMCCCAGGaAGAAGCCATAGCCCCAC-TATCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGACGCTTATTAATATAGTTCCATAAAAATCAAGAAC
TTTATCAGTATTAAATTTCCAAAAAATCTAATATTTTAATACAGCTTTCTACTCMACATCCAATTTACATTTTACCTCCTACTAATTACACAGCAAAACACGTGATATAACCTTATGCGCTTGGAGTAC
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>C6_NY-C-NY1306-S_01
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ATAGGACATATTATGTATAATAGTACATAAATTAATGTATTAAGACATACTATGTATAATAGTACATTATATTATATGCCCCATGCTTATAAGCATGTACTTCTCATCATTTAAAGTACATAGTACAT
AATGTTGTTCATCGTACATAGCACATTAAGTCAAATCAGTCCTTGTCAACATGCGTATCCCGCCCCCTAGATCACGAGCTTAATTACCATGCCGCGTGAAACCAGCAACCCGCTGGGCAGGGATCCCTCTT
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ACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACATTTGGTATTTTTAATTTTTGGGGGGATGCTTGGGACTCAGCA
>NY-C-total
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>NY-D-toyal
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CATAGTTAATGTAGCTTAAaCCAGGCAAAGCAAAAN
>D6_NY-D-NY1306-S
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AAACACGTGATATAACCTTATGCGCTCGTAGTACATAAAATCAATGTGCTAGGACATGCATGTATAACAGTACATGAGTTAGCGTATAGGACATATTATGCATAATAGTACATAAATTAATGTATTAA
GACATACTATGTATAATAGTACATTATATTATATGCCCCATGCTTATAAGCATGTACTTCTCACCATTTAAAGTACATAGTACATAGTGTTGTTCATCGTACATAGCACATTAAGTCAAATCAGTCCTC
GTCAACATGCGTATCCCGTCCCCTAGATCACGAGCTTAATTACCATGCCGCGTGAAACCAGCAACCCGCTGGGCAGGGGATCCCTCTTCTCGCTCCGGGCCCATGAACCGTGGGGGTAGCTATTTCATGAAC
TTTATCAGACATCTGGGTCTTTTTTCAGGGCCATCTCACCTAAAATCGCCCACTCCTTGCAATATAAGACATCTCGATGGACTAATGACTAATCAGCCCATGCTCACACATAACTGGGGGGGCAT
>NY-E-total
AGnAg-AGCCATAGCCCCdCAT-ATCaA-CACCCAAAGCTG-AAGTTCTATTTAAACTATTCCCTGACGCTTATTAATATAGTTCCATAAAAATCAAGAACTTTATCAGTATTAAATTTCCAAAAAATTTA
ATATTTTAATACAGCTTTCTACTCMACATCCAATTTACATTTTATGTCCYACTAATTACMCAGCAAAACACRTGATATAACCTTATGCGCTCGTAGTACATAAAATCAATGKGCTAGGACATGCATGTA
TAACAGTACATGAGTTAGCGTATAGGACATRTTATGCATAATAGTACATAAATTAATGTATTAAGACATACTATGTATAATAGTACATTATATTAT-ATGCCCCATG-C-TTATAAGCATGTACTTCTC
ACCATTTAAAGTACATAGTACATAGTGTTGTTCATCGTACAT-AGCACATTAAGTCAAATCAGTCCTCGTCAACATGCGTATCCCGTCCCCTAG-ATCACGAGCTTAATTACCATGCCGCGTGAAACCAGC
AACCCGCTGGGCA-GGG-ATCCCTC-TTCTCGCTCCGGGCCCATGAACCGTGGGGGT--AGCTATTTCATGAACTTTATCAGACATCTGGTTCTTTTTTCAGGGCCATCTCA--CCTA--AA-ATCGCCCACTC
CTTGC-AATATAAGA-CATCTCGAT-GGACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACATTTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCAATGGCCGTCTGAGG
CCCCGTCCCGGAGCATGAATTGTAGCTGGACTTAACTGCATCTTGAGCATCCCCATAATGGTAGGGGCRGGGCATTGCAGTCAAKGGTCACAGGACATAATTATTATTTCATGAGTCAACCCTAAGATCTA
TTTTCCCCCCCCTTCTTATTTTTTCCCCCTTATATAGTTATCACCATTTTTAACACACTTTCCCCTAGATATAA-TTTrAAATTtATCACATTTCCAATACTCAAAATAGCACTCCAGAGGGAGGTAAGTA
TATAAACGCCAATTTTTCCCTAATTATGCATAGTTAATGTAGCTTAACCAGGCCAAAAGCAAAA
>E5_NY-E-NY1306-S
AGCTGAAGTTCTATTTAAACTATTCCCTGACGCTTATTAATATAGTTCCATAAAAATCAAGAACTTTATCAGTATTAAATTTCCAAAAAATTTAATATTTTAATACAGCTTTCTACTCAACATCCAAT
TTACATTTTATGTCCTACTAATTACACAGCAAAACACATGATATAACCTTATGCGCTCGTAGTACATAAAATCAATGTGCTAGGACATGCATGTATAACAGTACATGAGTTAGCGTATAGGACATATTA
TGCATAATAGTACATAAATTAATGTATTAAGACATACTATGTATAATAGTACATTATATTATATGCCCCATGCTTATAAGCATGTACTTCTCACCATTTAAAGTACATAGTACATAGTGTTGTTCATC
GTACATAGCACATTAAGTCAAATCAGTCCTCGTCAACATGCGTATCCCGTCCCCTAGATCACGAGCTTAATTACCATGCCGCGTGAAACCAGCAACCCGCTGGGCAGGGATCCCTCTTCTCGCTCCGGGCCCAT

Fig. 1. Sequences of 4 antler samples. B5_NY-B-NY1306-S and NY-B-total from NY-B, C6_NY-C-NY1306-S_01 and NY-C-total from NY-C, D6_NY-D-NY1306-S and NY-D-total from NY-D, E5_NY-E-NY1306-S and NY-E-total from NY-E. The other letters than A, G, C and T mean heterozygotic sites (IUPAC code). V is A/C/G. H is A/C/T. D is A/G/T. B is C/G/T. M is A/C. R is A/G. W is A/T. S is C/G. Y is C/T. K is G/T. N is any of the four bases (G/A/T/C). In the symbol the capital letters indicate that they are the main bases at each position, and the small letter means that it is the second main at the position.

Jehle et al [5], based on the observed sequence distances, proposed to define that two (or more isolates) belong to the same species if the Kimura 2-parameter distance between single and/or concatenated nucleotide sequences is smaller than 0.015. Furthermore, two viruses should be considered as different virus species if the distance between single and/or concatenated sequences is larger than 0.050. For distances between 0.015 and 0.050, complementary information is needed to determine whether two viruses are the same or different species.

Genetic identification of traditional Korean herbs should help to ensure the safeuse of traditional Korean herbal materials, so the method for identifying the origin is very important. Results of this study leave more to be investigated and answered, as in our study we couldn't identify rearing places or areas of Cervus elaphus species. Nevertheless, they offer a useful tool for identification of *Cervus* species. In conclusion, these results suggest that genetic distance methods might be able to provide the identification of *Cervus* species.

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