



Identification of the prion-related protein gene (*PRNT*) sequences in various species of the Cervidae family

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Abstract

Chronic wasting disease (CWD) is caused by abnormal deleterious prion protein (PrP^{Sc}), and transmissible spongiform encephalopathy occurs in the Cervidae family. In recent studies, the susceptibility of prion disease has been affected by polymorphisms of the prion gene family. However, the study of the *prion-related* protein gene (*PRNT*) is rare, and the DNA sequence of this gene was not fully reported in all Cervidae families. In the present study, we amplified and first identified *PRNT* DNA sequences in the Cervidae family, including red deer, elk, sika deer and Korean water deer, using polymerase chain reaction (PCR). We aligned nucleotide sequences of the *PRNT* gene and the amino acid sequences of prion-related protein (Prt) protein among several species. In addition, we performed phylogenetic analysis to measure the evolutionary relationships of the *PRNT* gene in the Cervidae family. Furthermore, we performed homology modeling of the Prt protein using SWISS-MODEL and compared the structure of Prt protein between sheep and the Cervidae family using the Swiss-PdbViewer program. We obtained much longer *PRNT* sequences of red deer compared to the *PRNT* gene sequence registered in GenBank. Korean water deer denoted more close evolutionary distances with goats and cattle than the Cervidae family. We found 6 Cervidae family-specific amino acids by the alignment of Prt amino acid sequences. There are significantly different distributions of hydrogen bonds and the atomic distance of the N-terminal tail and C-terminal tail between sheep and the Cervidae family. We also detected the mRNA expression of *PRNT* gene in 3 tissues investigated. To our knowledge, this report is the first genetic study of the *PRNT* gene in the Cervidae family.

Keywords Deer · Prion · Prion protein gene · *PRNP* · Prion-related protein gene · *PRNT* · Cervidae

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Introduction

Prion diseases are caused by misfolded prion protein (PrP^{Sc}) and denote paramount infection ranges and include various types, such as Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in elk and deer [1–9]. Recent studies have reported that the susceptibility of prion disease was affected by polymorphisms of the prion gene family, including prion protein gene (*PRNP*), prion-like protein gene (*PRND*) and shadow of prion protein (*SPRN*) [10–18]. Although the exact underlying mechanism on the relationship between polymorphisms and susceptibility to prion diseases was elusive, prion disease-related polymorphisms located on 3' untranslated region (UTR) of the *SPRN* and *PRND* genes were presumed to be related to regulate the expression of the genes by micro RNA [11, 17]. However, the study of the last member of the prion gene

family, prion-related protein gene (*PRNT*), is rare, and even the DNA sequence of this gene has not been fully specified in the major host of prion disease such as elk, sika deer and water deer.

In previous studies, the *PRNT* gene was investigated in mainly ruminants. In sheep, prion-related protein (Prt) was detected in mainly reproductive organs and ejaculated spermatozoa, and the inhibition of Prt reduced the reproductive capacity of sperm [19, 20]. In addition, the *PRNT* gene was highly polymorphic in Portuguese sheep and Korean native black goats, and polymorphisms of the *PRNT* gene were significantly related to growth traits in Chinese and Mongolian sheep [21–23]. However, there was no single nucleotide polymorphism (SNP) in the open reading frame (ORF) of the *PRNT* gene in Hanwoo and Holstein raised in Korea [24]. Interestingly, recent studies provided evidence for the association of prion disease with the *PRNT* gene by strong genetic linkage disequilibrium (LD) among SNPs of *PRNP*, *PRND* and *PRNT* genes in sheep and goats, which are well known as hosts of prion disease [13, 23, 25]. However, except for red deer (*Cervus elaphus*), the DNA sequences of the *PRNT* gene have not been reported in the Cervidae family thus far.

In the present study, we designed *PRNT* gene-specific primers based on the *PRNT* gene sequence of the sheep (*Ovis aries*) registered in GenBank and amplified the *PRNT* sequence in the Cervidae family, including red deer, elk (*Cervus canadensis*), sika deer (*Cervus nippon hortulorum*) and water deer (*Hydropotes inermis argyropus*) using polymerase chain reaction (PCR) and identified the DNA sequences of the *PRNT* gene. In addition, we aligned the nucleotide sequences of the *PRNT* gene and the amino acid sequences of the Prt protein among several species and performed neighbor-joining method-based phylogenetic analysis using Molecular Evolutionary Genetics Analysis (MEGA) X software [26, 27]. Furthermore, we performed homology modeling of the Prt protein using SWISS-MODEL and compared the structure of Prt protein using Swiss-PdbViewer programs [28, 29]. We also investigated

mRNA expression of *PRNT* gene according to tissue types using reverse transcription (RT)-PCR.

Materials and methods

Ethical statement

Animal samples of 5 red deer, 5 elk, 5 sika deer and 2 Korean water deer were collected from animal farms in Gyeongsangnam-do, Republic of Korea. All experimental procedures and animal care were approved according to the recommendations of the Institutional Animal Care and Use Committee of Jeonbuk National University (IACUC Number: CBNU-2019–0076), and all efforts were made to minimize suffering. All experiments were performed in accordance with the Korea Experimental Animal Protection Act.

Genomic DNA (gDNA) extraction

The gDNA was isolated from 20 mg brain tissues of 5 red deer, 5 elk, 5 sika deer and 2 Korean water deer using a QIAamp DNA Mini Kit (Qiagen, USA) following the manufacturer's instructions. Detailed information on the animal samples used in the present study is described in Table 1.

PCR

PCR was performed with gene-specific forward and reverse primers. Detailed information of primers and experimental conditions were described in Table 2. The PCR reagents contained 25 pmol of each primer, 5 µl of 10× *Taq* DNA polymerase buffer, 1 µl of 10 mM dNTPs and 2.5 units of *Taq* DNA polymerase (Promega, USA). The PCR conditions were as follows by manufacturer's instructions. The S-1000 Thermal Cycler (Bio-Rad Laboratories, USA) was used.

Table 1 Detailed information on the prion-related protein gene (*PRNT*) sequences analyzed in this study

Scientific name	Common name	GenBank numbers	
		Nucleotides	Amino acids
<i>Ovis aries</i>	Sheep	EF397417.1	NP_001091118.1
<i>Capra hircus</i>	Goat	AM412782.1	CAL85353.1
<i>Bos taurus</i>	Cattle	DQ205538.1	ABB51166.1
<i>Cervus elaphus</i>	Red deer (GenBank)	EF397422.1	ABO86201.1
<i>Cervus elaphus</i>	Red deer	In this study	In this study
<i>Cervus canadensis</i>	Elk	In this study	In this study
<i>Cervus nippon hortulorum</i>	Sika deer	In this study	In this study
<i>Hydropotes inermis argyropus</i>	Korean water deer	In this study	In this study

Table 2 Detailed information of primers used in this study

Target	Primers		Annealing temperature (°C)	Product size
	Forward	Reverse		
<i>PRNT</i>	ACTGGCCCTCTCTGGAAA	TCTGGCTGGGGAAAGACT	62	590 bp
<i>PRNT</i> (RT-PCR)	TCCATTTCTCAACAGACTGAGA	ACTCTATCACATGCCTGACC	56	101 bp
<i>ACTB</i> (RT-PCR)	GCTGTCCCTGTATGCCTCTG	TTTCTGCATCCTGTCTGCGAT	58	529 bp (cDNA) 615 bp (gDNA)

Amplicon sequencing

A 5 µl aliquot of the PCR product was analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide (EtBr). The purification of PCR products for DNA sequencing was performed using a QIAquick Gel Extraction Kit (Qiagen, USA). The PCR products were sequenced on an ABI 3730 automatic sequencer using a *Taq* Dideoxy Terminator Cycle Sequencing Kit (ABI, USA) using same Forward or Reverse primers used to perform PCR reaction. Genotyping was performed using Finch TV software (Geospiza Inc., Seattle, USA).

Evolutionary relationships of taxa

DNA sequences of the ORF of the *PRNT* gene in the Cervidae family were used for phylogenetic analysis. A neighbor-joining phylogenetic tree was constructed using MEGA X software. The bootstraps test from 1000 replicates was applied to estimate the confidence level of the branching patterns of the neighbor-joining tree.

Sequence prediction and comparison of the Prt protein

Amplicons of the *PRNT* gene in the Cervidae family were analyzed by the web-based translate tool (<https://web.expasy.org/translate/>). Amino acid sequence alignment was performed using ClustalW2 (<https://www.ebi.ac.uk/Tools/msa/clustalw2/>). Protein sequences of Prt were obtained from GenBank at NCBI. Detailed information is described in Table 1.

Modeling and structure comparison of Prt protein

Models were built by using the SWISS-MODEL program (<https://swissmodel.expasy.org/>). The SWISS-MODEL repository is a database of annotated 3D protein structure models generated by the SWISS-MODEL homology-modeling pipeline. The transcript with the reference sequences NP_001091118.1, and the deer Prt sequence was used for homology modeling. The SWISS-MODEL program could

not build modeling of caprine and bovine Prt protein (data not shown). After homology-based modeling, the Swiss-PdbViewer program (<https://spdbv.vital-it.ch/>) was utilized to analyze the hydrogen bonds and atomic distance of the Prt protein.

RNA extraction

Tissue samples from a sika deer were collected, immediately frozen at – 80 °C and stored prior to investigation. Tissue samples, including heart, kidney, uterus, brain and pancreas were homogenized in 1 ml of TRIZOL reagent (Thermo Fisher Scientific, USA) per 100 mg of tissue using homogenizer. After 5 min incubation, 200 µl of chloroform was added and mixed vigorously 15 s and incubated them at room temperature for 2 min. Samples were centrifuged at 12,000×g for 15 min at 4 °C and aqueous phase was collected. The RNA from the aqueous phase was precipitated by mixing with 500 µl of isopropyl alcohol and incubated them at room temperature for 10 min. Samples were centrifuged at 12,000×g for 10 min at 4 °C and the supernatant was removed. RNA pellet was washed twice with 1 ml of 75% ethanol. Samples were centrifuged at 12,000×g for 10 min at 4 °C and the supernatant was removed and RNA pellet was air-dried for 10 min. RNA is retrieved in 50 µl of DEPC-treated water.

Complementary DNA (cDNA) synthesis

To exclude gDNA contamination prior to cDNA synthesis, we removed gDNA from 1 µg RNA with 1 unit of DNase I (Thermo Fisher Scientific, USA). RNA quality and gDNA contamination in cDNA were checked by the amplification (615 bp) of the *ACTB* gene with primers encompassing an intron (Table 2). The cDNA synthesis was performed by SMOBIO #RP1400S ExcelRT™ Reverse Transcription Kit II (SMOBIO, Taiwan). The cDNA synthesis conditions were as follows: 25 °C for 10 min to incubate, 37 °C for 50 min to incubate and 85 °C for 5 min to terminate the reaction. The S-1000 Thermal Cycler (Bio-Rad Laboratories, USA) was used.

Results

Identification of the *PRNT* sequences of the Cervidae family

We designed *PRNT* gene-specific primers based on the *PRNT* gene sequence of the sheep (*Ovis aries*) registered in GenBank (Gene ID: EF397417.1) to amplify the *PRNT* gene of the Cervidae family. We performed PCR using *PRNT* gene-specific primers and gDNA of

the Cervidae family and obtained amplicons composed of 590 bp encompassing the ORF of the *PRNT* gene in the Cervidae family. All animal samples of each cervid breed showed identical *PRNT* gene sequences. Identified gDNA sequences of the *PRNT* gene of the Cervidae family aligned in Fig. 1. In the present study, we first identified *PRNT* gene sequences of elk, sika deer and Korean water deer. In addition, we obtained much longer sequences containing adjacent region of *PRNT* gene of red deer (590 bp) compared to the *PRNT* gene sequence (260 bp) registered in GenBank (EF397422.1). Interestingly, the *PRNT* gene

Fig. 1 Comparison of prion-related protein gene (*PRNT*) gene sequences in the Cervidae family. Sequence alignment includes red deer (*Cervus elaphus*, EF397422.1), red deer (*Cervus elaphus*, in this study), elk (*Cervus canadensis*, in this study), sika deer (*Cervus nippon hortulorum*, in this study) and Korean water deer (*Hydropotes inermis argyropus*, in this study). Detailed information for the *PRNT* gene sequences described in Table 1. Nucleotide sequences were aligned using ClustalW2. Asterisks indicate identical nucleotides among the Cervidae family. (Color figure online)

	Primer-F	Start codon	
<i>Cervus elaphus</i> (EF397422.1)	ACTGGCCCTCTCTGGAAACCTTTCTTGCTAGTGCCCATGSGTAGACAGAACCTTCCCAT	ATG	39
<i>Cervus elaphus</i> (In this study)	ACTGGCCCTCTCTGGAAACCTTTCTTGCTAGTGCCCATGSGTAGACAGAACCTTCCCAT	ATG	60
<i>Cervus canadensis</i> (In this study)	ACTGGCCCTCTCTGGAAACCTTTCTTGCTAGTGCCCATGSGTAGACAGAACCTTCCCAT	ATG	60
<i>Cervus nippon hortulorum</i> (In this study)	ACTGGCCCTCTCTGGAAACCTTTCTTGCTAGTGCCCATGSGTAGACAGAACCTTCCCAT	ATG	60
<i>Hydropotes inermis argyropus</i> (In this study)	ACTGGCCCTCTCTGGAAACCTTTCTTGCTAGTGCCCATGSGTAGACAGAACCTTCCCAT	ATG	60
***** * * *****			
<i>Cervus elaphus</i> (EF397422.1)	TTGCTCAGTCATTATCCATTTTCCTCAACAGACTGAGACCTGGAAGGCAGCAGTGTCAATT		99
<i>Cervus elaphus</i> (In this study)	TTGCTCAGTCATTATCCATTTTCCTCAACAGACTGAGACCTGGAAGGCAGCAGTGTCAATT		120
<i>Cervus canadensis</i> (In this study)	TTGCTCAGTCATTATCCATTTTCCTCAACAGACTGAGACCTGGAAGGCAGCAGTGTCAATT		120
<i>Cervus nippon hortulorum</i> (In this study)	TTGCTCAGTCATTATCCATTTTCCTCAACAGACTGAGACCTGGAAGGCAGCAGTGTCAATT		120
<i>Hydropotes inermis argyropus</i> (In this study)	TTGCTCAGTCATTATCCATTTTCCTCAACAGACTGAGACCTGGAAGGCAGCAGTGTCAATT		120
***** * * *****			
<i>Cervus elaphus</i> (EF397422.1)	CTTTCACCTGTGGTATCTGCAGAGCCCTGGGTATGGTCAGGCATGTGATAGAGAGTCAGTA		159
<i>Cervus elaphus</i> (In this study)	CTTTCACCTGTGGTATCTGCAGAGTCCTGGGTATGGTCAGGCATGTGATAGAGAGTCAGTA		180
<i>Cervus canadensis</i> (In this study)	CTTTCACCTGTGGTATCTGCAGAGTCCTGGGTATGGTCAGGCATGTGATAGAGAGTCAGTA		180
<i>Cervus nippon hortulorum</i> (In this study)	CTTTCACCTGTGGTATCTGCAGAGCCCTGGGTATGGTCAGGCATGTGATAGAGAGTCAGTA		180
<i>Hydropotes inermis argyropus</i> (In this study)	CTTTCACCTGTGGTATCTGCAGAGCCCTGGGTATGGTCAGGCATGTGATAGAGAGTCAGTA		180
***** * * *****			
Stop codon			
<i>Cervus elaphus</i> (EF397422.1)	AAGATTTATTGCTAATGTATCCTAAACTCCAGTGGAGGCCAGACGACATCTCTCTCTCG		219
<i>Cervus elaphus</i> (In this study)	AAGATTTATTGCTAATGTATCCTAAACTCCAGTGGAGGCCAGATGCCATCTCTCTCTCG		240
<i>Cervus canadensis</i> (In this study)	AAGATTTATTGCTAATGTATCCTAAACTCCAGTGGAGGCCAGATGCCATCTCTCTCTCG		240
<i>Cervus nippon hortulorum</i> (In this study)	AAGATTTATTGCTAATGTATCCTAAACTCCAGTGGAGGCCAGATGCCATCTCTCTCTCG		240
<i>Hydropotes inermis argyropus</i> (In this study)	AAGATTTATTGCTAATGTATCCTAAACTCCAGTGGAGGCCAGATGCCATCTCTCTCTCG		240
***** * * *****			
<i>Cervus elaphus</i> (EF397422.1)	CTCTGGACTTGCACCTTCCCAAGCCACGTACCCTGGGTGT		260
<i>Cervus elaphus</i> (In this study)	CTCTGGACTTGCACCTTCCCAAGCCACGTACCCTGGGTGTCCATCTGCATGTGAGGAGA		300
<i>Cervus canadensis</i> (In this study)	CTCTGGACTTGCACCTTCCCAAGCCACGTACCCTGGGTGTCCATCTGCATGTGAGGAGA		300
<i>Cervus nippon hortulorum</i> (In this study)	CTCTGGACTTGCACCTTCCCAAGCCACGTACCCTGGGTGTCCATCTGCATGTGAGGAGA		300
<i>Hydropotes inermis argyropus</i> (In this study)	CTCTGGACTTGCACCTTCCCAAGCCACGTACCCTGGGTGTCCATCTGCATGTGAGGAGA		300
** *****			
<i>Cervus elaphus</i> (In this study)	GAGATCTAGAAGGCAGTCTCAATCATCGATAGCCCTCAACTGAAATGACTTCATCTCT		360
<i>Cervus canadensis</i> (In this study)	GAGATCTAGAAGGCAGTCTCAATCATCGATAGCCCTCAACTGAAATGACTTCATCTCT		360
<i>Cervus nippon hortulorum</i> (In this study)	GAGATCTAGAAGGCAGTCTCAATCATCGATAGCCCTCAACTGAAATGACTTCATCTCT		360
<i>Hydropotes inermis argyropus</i> (In this study)	GAGATCTAGAAGGCAGTCTCAATCATCGATAGCCCTCAACTGAAATGACTTCATCTCT		360
***** * * *****			
<i>Cervus elaphus</i> (In this study)	GACTTGACAGGGCTACAAAATTGCCTATGGAATGAATTAATATGTGTAAGTTTATAAAT		420
<i>Cervus canadensis</i> (In this study)	GACTTGACAGGGCTACAAAATTGCCTATGGAATGAATTAATATGTGTAAGTTTATAAAT		420
<i>Cervus nippon hortulorum</i> (In this study)	GACTTGACAGGGCTACAAAATTGCCTATGGAATGAATTAATATGTGTAAGTTTATAAAT		420
<i>Hydropotes inermis argyropus</i> (In this study)	GACTTGACAGGGCTACAAAATTGCCTATGGAATGAATTAATATGTGTAAGTTTATAAAT		420
***** * * *****			
<i>Cervus elaphus</i> (In this study)	TTAAATTAGTTGCTTGGAAACAGTCACAAATGACTATGCACATCTCTCTCCCTCCACA		480
<i>Cervus canadensis</i> (In this study)	TTAAATTAGTTGCTTGGAAACAGTCACAAATGACTATGCACATCTCTCTCCCTCCACA		480
<i>Cervus nippon hortulorum</i> (In this study)	TTAAATTAGTTGCTTGGAAACAGTCACAAATGACTATGCACATCTCTCTCCCTCCACA		480
<i>Hydropotes inermis argyropus</i> (In this study)	TTAAATTAGTTGCTTGGAAACAGTCACAAATGACTATGCACATCTCTCTCCCTCCACA		480
***** * * *****			
<i>Cervus elaphus</i> (In this study)	GTCAGCTGCTAATTTGAGACTAGCCAGGTATACTAATCACGTTTTTGAAAAATTCGCAT		540
<i>Cervus canadensis</i> (In this study)	GTCAGCTGCTAATTTGAGACTAGCCAGGTATACTAATCACGTTTTTGAAAAATTCGCAT		540
<i>Cervus nippon hortulorum</i> (In this study)	GTCAGCTGCTAATTTGAGACTAGCCAGGTATACTAATCACGTTTTTGAAAAATTCGCAT		540
<i>Hydropotes inermis argyropus</i> (In this study)	GTCAGCTGCTAATTTGAGACTAGCCAGGTATACTAATCACGTTTTTGAAAAATTCGCAT		540
***** * * *****			
Primer-R			
<i>Cervus elaphus</i> (In this study)	ATTTTGTGTTTTGATATCCTAAAAATACTTTCTGGCTGGGGAAAGACT		590
<i>Cervus canadensis</i> (In this study)	ATTTTGTGTTTTGATATCCTAAAAATACTTTCTGGCTGGGGAAAGACT		590
<i>Cervus nippon hortulorum</i> (In this study)	ATTTTGTGTTTTGATATCCTAAAAATACTTTCTGGCTGGGGAAAGACT		590
<i>Hydropotes inermis argyropus</i> (In this study)	ATTTTGTGTTTTGATATCCTAAAAATACTTTCTGGCTGGGGAAAGACT		590
***** * * *****			

sequences of the red deer identified in this study showed 3 mismatches and 98.84% sequence homology (257/260) with those of the red deer registered in GenBank. The *PRNT* gene sequences of the red deer identified in this study denoted 2 mismatches with those of the elk and 99.66% sequence homology (588/590). The *PRNT* gene sequences of sika deer showed 4 mismatches and 99.32% sequence homology with those of the red deer identified in this study (586/590). Notably, the *PRNT* gene sequences of the water deer showed 20 mismatches and 96.61% sequence homology with those of the red deer identified in this study (570/590). Interestingly, except for water deer, the start codon is conserved in all Cervidae families.

Phylogenetic analysis of the *PRNT* gene in the Cervidae family

To investigate the evolutionary relationships of taxa, the *PRNT* gene sequences in sheep, goats, cattle, red deer (GenBank), red deer (in this study), elk (in this study), sika deer (in this study) and Korean water deer (in this study) were analyzed by MEGA X. There were a total of 159 positions in the final dataset. The optimal tree of the *PRNT* gene in the Cervidae family with the sum of branch length = 0.04539976 is shown. The percentage of replicate trees in the bootstrap test (1000 replicates) is shown next to the branches [30]. The branch lengths indicate evolutionary distances and computed using the maximum composite likelihood method with units of the number of base substitutions per site. Notably, Korean

water deer showed closer evolutionary distances with goats and cattle than with the Cervidae family (Fig. 2).

Prediction and comparison of the Prt protein sequence in the Cervidae family

PRNT gene sequences of the Cervidae family were analyzed by a web-based translation tool (<https://web.expasy.org/translate/>). Notably, Korea water deer did not show a species-conserved start codon (Fig. 1), and 7 ORFs in three different possible reading frames were predicted. Detailed information is described in Fig. 3. Except for Korean water deer, all Cervidae families investigated in the present study showed identical Prt protein sequences. We aligned Prt sequences among several species and found that the Cervidae family showed 6 conserved amino acids of the Prt sequence (Fig. 4).

Homology modeling and structure comparison of Prt protein

We performed modeling of the Prt protein in sheep and the Cervidae family. A homology-modeling pipeline based on a SWISS-MODEL of the ovine and cervid Prt protein is described in Fig. 5. Ovine Prt protein was modeled based on 4g54.1. A template and cervid Prt protein were modeled based on 1jju.1. The template and 3D structure of Prt proteins are described in Fig. 5a, b. Ovine Prt protein showed an alpha helix in 5–12 and 26–27 residues and a close distance (black dotted line) between the N-terminal tail and the

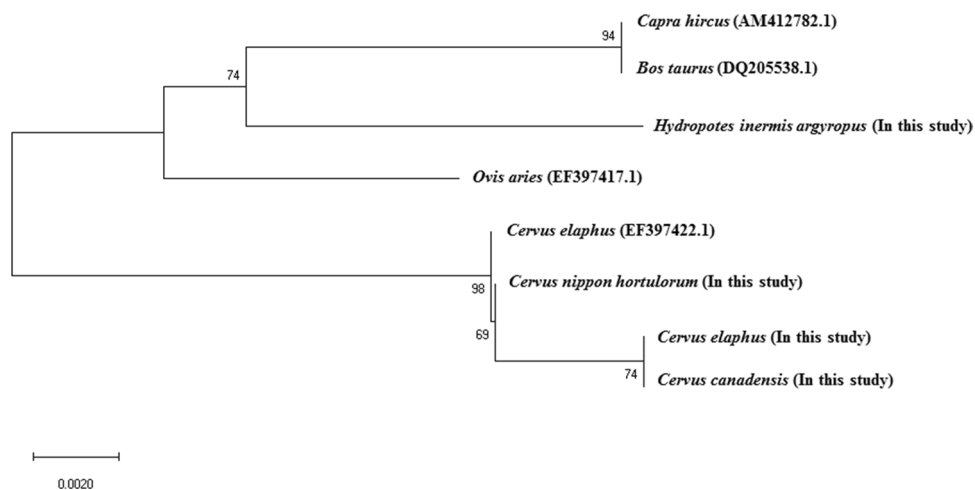


Fig. 2 Phylogenetic analysis of the *PRNT* gene in 8 species. Phylogenetic analysis includes sheep (*Ovis aries*, EF397417.1), goats (*Capra hircus*, AM412782.1), cattle (*Bos taurus*, DQ205538.1), red deer (*Cervus elaphus*, EF397422.1), red deer (*Cervus elaphus*, in this study), elk (*Cervus canadensis*, in this study), sika deer (*Cervus nippon hortulorum*, in this study) and Korean water deer (*Hydropotes inermis argyropus*, in this study). A phylogenetic tree was drawn

by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) X software. The numbers at the branch nodes represent bootstrap confidence intervals obtained from 1,000 replicates. The branch length of the tree denotes evolutionary distances, which were calculated using the Poisson correction method and are expressed in units of the number of amino acid substitutions per site. The scale bar corresponds to a distance of 0.0020

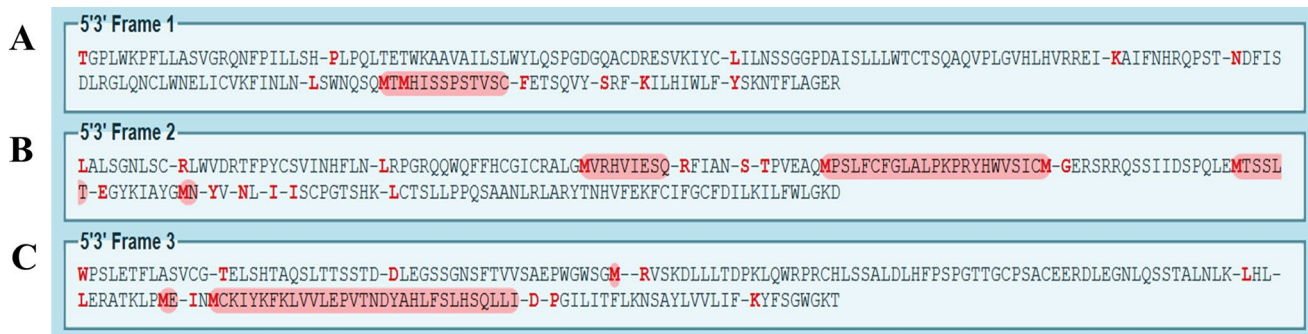


Fig. 3 Sequence prediction of *prion-related protein* (Prt) in Korean water deer. The amplicons of the *PRNT* gene in Korean water deer identified in this study were analyzed by a web-based translation tool

(<https://web.expasy.org/translate/>). Three panels (a, b and c) indicate predicted results of possible translated frames. Open reading frames (ORFs) are highlighted in red. (Color figure online)

<i>Ovis aries</i> (NP_001091118.1)	MGRQNSPILLSHYPLPQQTETWKA AVAILSLWYLQSPGDGQACDRESVKIYC	52
<i>Capra hircus</i> (CAL85353.1)	MGRQNFPIILLSHYPLPQQTETWKA AVAIISLWYLQSPGDGQACDRESVKIYC	52
<i>Bos taurus</i> (ABB51166.1)	MGRQNFPIILLSHYPLPQQTETWKA AVAIISLWYLQSPGDGQACDRESVKIYC	52
<i>Cervus elaphus</i> (In this study)	MGRQNFPIILLSHYFPFQQTETWKA AVSILSLWYLQSPGYGQACDRESVKIYC	52
<i>Cervus canadensis</i> (In this study)	MGRQNFPIILLSHYFPFQQTETWKA AVSILSLWYLQSPGYGQACDRESVKIYC	52
<i>Cervus nippon hortulorum</i> (In this study)	MGRQNFPIILLSHYFPFQQTETWKA AVSILSLWYLQSPGYGQACDRESVKIYC	52
	* * * * **	

Fig. 4 Comparison of Prt amino acid sequences in sheep, goats, cattle, red deer, sika deer and Korean water deer. Prt protein sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI), including those of sheep (*Ovis aries*, NP_001091118.1), goats (*Capra hircus*, CAL85353.1), and cattle (*Bos taurus*, ABB51166.1). Prt protein sequences of red deer, elk

and sika deer were obtained from this study. Protein sequences were aligned using ClustalW2. Colors indicate the chemical properties of amino acids; blue: acidic; blue: small and hydrophobic, magenta: basic, green: hydroxyl, sulfhydryl, amine and glycine. Asterisks indicate differences in amino acids between the Cervidae family and other species. (Color figure online)

C-terminal tail (Fig. 5a). However, cervid Prt protein showed an alpha helix in 21–32 and 39–41 residues and a relatively long distance (black dotted line) between the N-terminal tail and the C-terminal tail compared to the ovine Prt protein (Fig. 5b). In addition, the Swiss-PdbViewer program (<https://spdbv.vital-it.ch/>) was utilized to analyze the hydrogen bonds of the Prt proteins and measure the distances of the N-terminal tail and C-terminal tail of ovine and cervid Prt proteins. Notably, the distribution of hydrogen bonds (green dotted lines) was significantly different between ovine Prt protein (Fig. 5c) and cervid Prt protein (Fig. 5d). Furthermore, the distances of the N-terminal tail and C-terminal tail (between Tyr13 and Pro37 residues) were 3.88 Å (yellow dotted line) in ovine Prt protein (Fig. 5c). The distances of the N-terminal tail and C-terminal tail (between Trp22 and Gln41 residues) showed 31.74 Å (yellow dotted line) in cervid Prt protein (Fig. 5d).

The mRNA expression of *PRNT* gene according to tissue types in deer

To investigate mRNA expression of the *PRNT* gene according to tissue types in deer, we extracted RNA from heart,

kidney, uterus, brain and pancreas of a sika deer. The gDNA contamination was double checked by amplification of the *ACTB* gene with a primer combination encompassing an intron (Table 2). A product (615 bp) of the *ACTB* gene from contaminated gDNA containing the intron makes slightly larger than the expected cDNA product (529 bp). The absence of 615 bp product from distilled water (DW) and cDNA used as a template was verified no contamination of gDNA in cDNA. To confirm mRNA expression of the *PRNT* gene, the experiments were performed in triplicate from different regions of each tissue. Notably, we observed mRNA expression of *PRNT* gene in heart, brain and pancreas (Fig. 6).

Discussion

CWD has been reported in extensive regions of the United States, Canada and Korea [31–33]. However, recent studies have reported that the number of CWD-reported countries is increasing [34, 35]. In addition, the possibility of cross-species transmission of CWD prion has been suggested in non-human primates and human prion transgenic mouse models

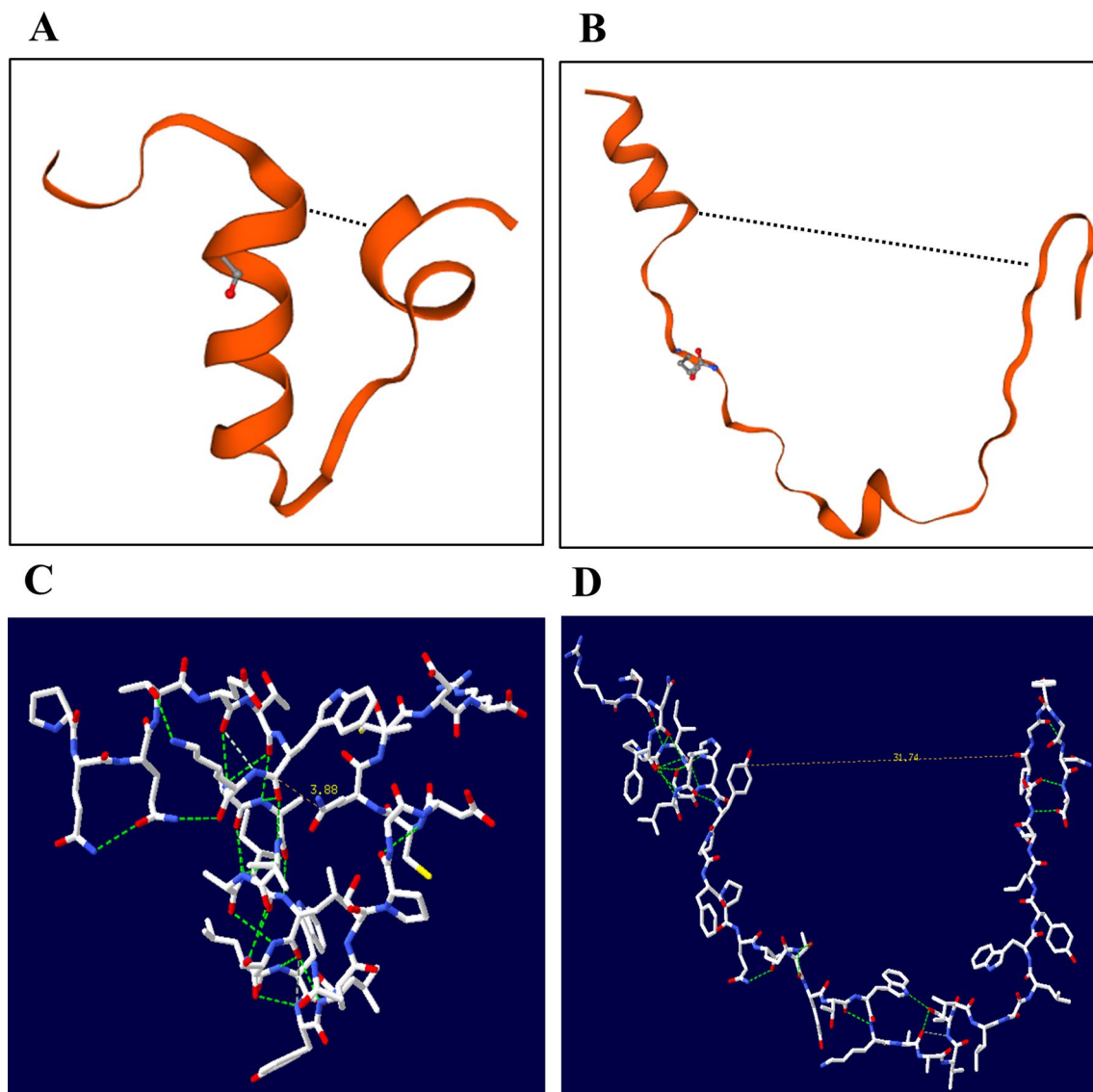


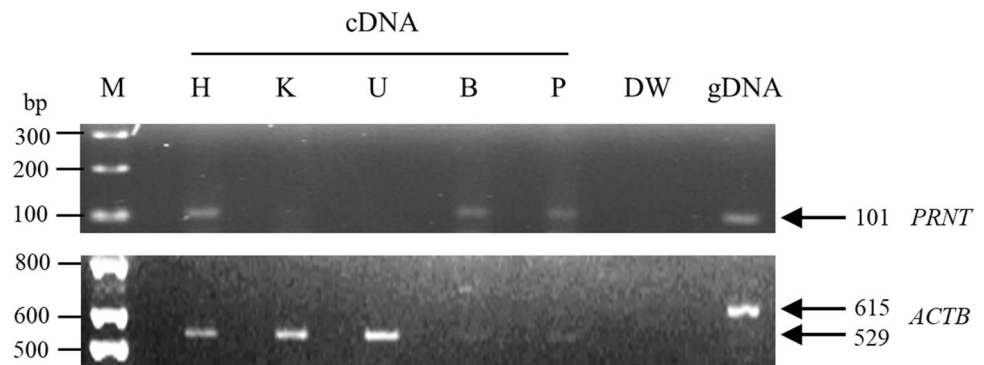
Fig. 5 Homology modeling and comparison of Prt protein between sheep and Cervidae family. **a** Homology-based structure prediction by the SWISS-MODEL program in ovine Prt protein. The black dotted line indicates the distance between the N-terminal tail and the C-terminal tail. **b** Homology-based structure prediction by the SWISS-MODEL program in the Prt protein of the Cervidae family. The black dotted line indicates the distance between the N-terminal tail and the C-terminal tail. **c** Analysis of hydrogen bonds and atomic distance in

ovine Prt protein. Green dotted lines indicate hydrogen bonds. Yellow dotted lines indicate distances of the N-terminal tail and C-terminal tail (between Tyr13 and Pro37 residues) and denotes 3.88 Å in ovine Prt protein. **d** Analysis of hydrogen bonds and atomic distance in Prt protein of the Cervidae family. Green dotted lines indicate hydrogen bonds. The yellow dotted line indicates the distances of the N-terminal tail and C-terminal tail (between Trp22 and Gln41 residues) and denotes 31.74 Å. (Color figure online)

[36]. Thus, the prevention of CWD is a very important issue, and an in-depth understanding of CWD-related genes is an important baseline study. Previous studies have reported that the SNPs of the *PRNT* gene showed strong LD with *PRNP* and *PRND* SNPs in sheep and goats, which are major hosts of scrapie. Portuguese sheep were the first to show a strong LD between *PRNP* codons 136, 154, 171 and codon 26 of the *PRND* gene [13]. In addition, heterozygotes (c.78G > A) of the *PRND* gene were significantly linked to three *PRNT* haplotypes [23]. Strong genetic LD among *PRNP* codon

143, *PRND* c.28 T > C, c.151A > G, c.385G > C and *PRNT* c.321 T > C SNPs showed in Korean native black goats [25]. However, among prion disease-resistant dogs, there was no strong LD among SNPs of prion gene family [37–39]. Since properties of LD among SNPs of prion gene family have been different between prion disease-susceptible and resistant animals, LD analysis in deer is highly desirable in the future. However, to date, because there is no evidence that the *PRNT* gene and/or Prt protein are directly linked to TSE disease, CWD infection study in overexpressed and

Fig. 6 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the *PRNT* gene according to tissue types in deer. The mRNA expression of *PRNT* gene was investigated in heart, kidney, uterus, brain and pancreas using *PRNT* and *ACTB* gene-specific primers. *M* DNA 100 bp ladder marker, *H* heart, *K* kidney, *U* uterus, *B* brain, *P* pancreas



knockout models of the *PRNT* gene are also needed in the future. We also observed mRNA expression of the *PRNT* gene in heart, brain and pancreas (Fig. 6). Since we investigated mRNA expression of *PRNT* gene in single breed, further investigation on mRNA expression of the *PRNT* gene in a larger number of various cervid breeds is needed in the future. Thus, the study of the association between the *PRNT* gene and CWD is highly desirable in the future. In the present study, we first reported *PRNT* gene sequences of the Cervidae family, including elk, sika deer and Korean water deer. In addition, we reported much longer sequences containing adjacent region of the *PRNT* gene of the red deer. Although we did not find the *PRNT* polymorphisms due to small sample size, investigation of the *PRNT* polymorphisms in larger samples is highly desirable in the future. Based on our sequence identification, further case–control association studies between CWD-infected animals and healthy animals are highly desirable to evaluate the susceptibility of CWD according to alleles of SNPs of the *PRNT* gene.

Among the Cervidae family, Korean water deer showed significantly different *PRNT* gene sequences compared to red deer with 20 mismatches (Fig. 1). Furthermore, we performed a phylogenetic analysis and found that Korean water deer showed more close evolutionary distances with goats and cattle, not the Cervidae family (Fig. 2). Interestingly, one mismatch was located on the start codon, in which conserved interspecies and Korean water deer showed significantly different Prt sequences compared to those of other species (Fig. 3). The possibility of infection of CWD to Korean water deer has been identified using protein misfolded cyclic amplification (PMCA); however, natural cases of CWD have not been reported in Korean water deer thus far. In addition, the lack of a start codon might imply that there is not protein translated from this genomic sequence in Korean water deer. Since the canonical pattern of the *PRNT* gene of the Cervidae family has not been identified in Korean water deer, further investigation of the relationship between the *PRNT* gene and susceptibility to CWD is needed in the future.

We also carried out the alignment of Prt protein sequences among sheep, goats, cattle and Cervidae families, including red deer, elk and sika deer (Fig. 4). Compared to ovine Prt protein, cervid Prt protein showed 6 specific amino acid residues. Since the Cervidae family-specific residues induced distinct Prt protein structure compared to ovine Prt protein, we performed homology modeling of Prt protein and compared protein structure. Notably, ovine Prt protein showed significantly different conformational structures compared to cervid Prt protein (Fig. 5). The distribution of hydrogen bonds is significantly different between these two Prt proteins, and the atomic distance between the N-terminal tail and the C-terminal tail of the cervid Prt protein was 8 times longer than that of the ovine Prt protein. Since Prt protein is associated with fertility and protein structure may affect protein function, further research is needed to confirm whether the structural difference between two proteins can induce functional differences in reproductive capacity.

Conclusion

In the present study, we first identified the *PRNT* sequences of red deer, elk, sika deer and Korean water deer. In addition, we obtained much longer sequences containing adjacent region of the *PRNT* gene of red deer compared to the *PRNT* gene sequences registered in GenBank. Using phylogenetic analysis, we calculated the evolutionary relationships of taxa among the Cervidae family. Notably, Korean water deer showed more close evolutionary distances with goats and cattle, not the Cervidae family. We compared Prt amino acid sequences among several species and found Cervidae family-specific amino acid residues. Finally, we compared the structure of Prt protein between sheep and the Cervidae family. We identified a significantly different distribution of hydrogen bonds and atomic distance between the N-terminal tail and the C-terminal tail. We observed mRNA expression of *PRNT* gene in 3 deer tissues tested.

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Author contributions ISR, YCK, HEK, HJS and BHJ conceived and designed the experiment. ISR, YCK, HJK, SYW and MJJ performed the experiments. ISR, YCK, HJK, SYW, MJJ, HEK, HJS and BHJ the data. ISR, YCK, HEK, HJS and BHJ wrote the paper. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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