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EDITED BY
Hua Wu,
Central China Normal University, China

REVIEWED BY
Rodrigo Morales,
University of Texas Health Science Center at Houston, United States
Yalçın Yaman,
Siirt University, Turkey
Justin J. Greenlee,
Agricultural Research Service (USDA), United States

*CORRESPONDENCE
Alfred L. Roca
roca@illinois.edu

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Extrapolating the susceptibility of Eld's deer (*Rucervus eldii thamin*) to chronic wasting disease from prion protein gene (*PRNP*) polymorphisms

Tolulope I.N. Perrin-Stowe[1,2], Yasuko Ishida[3], Dolores M. Reed[4], Emily E. Terrill[2], Oliver A. Ryder[5,6], Jan E. Novakofski[3,7], Nohra E. Mateus-Pinilla[3,7,8,9], Budhan S. Pukazhenthi[4] and Alfred L. Roca[1,3,7,9,10]*

[1]Program in Ecology, Evolution, and Conservation Biology, School of Integrative Biology, University of Illinois at Urbana-Champaign, Urbana, IL, United States, [2]School of Integrative Biology, University of Illinois at Urbana-Champaign, Urbana, IL, United States, [3]Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, United States, [4]Center for Species Survival, Smithsonian's National Zoo and Conservation Biology Institute, Front Royal, VA, United States, [5]San Diego Zoo Institute for Conservation Research, Escondido, CA, United States, [6]Department of Evolution, Behavior, and Ecology, Division of Biology, University of California San Diego, La Jolla, CA, United States, [7]Illinois Natural History Survey-Prairie Research Institute, Champaign, IL, United States, [8]Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, IL, United States, [9]Department of Natural Resources and Environmental Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, United States, [10]Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL, United States

Chronic wasting disease (CWD) is a prion disease of North American cervids. The transmission of CWD to endangered cervid species is of concern for captive breeding programs. Trans-species transmission could occur *via* direct contact with infected wild deer, or *via* prion contaminated fomites. Variation in the prion protein gene, *PRNP*, is associated with differences in CWD susceptibility among cervids. We therefore sequenced *PRNP* in 36 endangered Eld's deer (*Rucervus eldii thamin*), detecting five synonymous and two non-synonymous SNPs. Three haplotypes were inferred, suggesting that genetic management in captive breeding programs has been effective at maintaining *PRNP* diversity. The haplotypes encoded two PrP protein variants. The more common Eld's deer PrP variant encodes methionine at codon 208 and glutamine at codon 226. Because this protein variant is identical to a common PrP variant in white-tailed deer and mule deer and is especially common in white-tailed deer positive for CWD, we recommend reducing the frequency of this variant in the breeding stock, while implementing strict management practices to avoid exposure to wild North American cervids. The frequency of the other PrP variant, which differs from variants present in these North American cervids, was low. It has the potential to reduce susceptibility to CWD and thus could be increased in frequency. While *PRNP* haplotype frequencies should be shifted, genetic diversity should be maintained. Ultimately protein diversity may be protective should CWD infect

the species, and trans-species polymorphisms are suggestive of past balancing selection and a potential fitness advantage for *PRNP* diversity.

KEYWORDS

brow-antlered deer, cervids, prion, thamin, transmissible spongiform encephalopathy

Introduction

The genetic management of endangered species in captive-breeding programs has been an important aspect of species conservation (Ballou, 1992). Maximum genetic diversity is maintained in the captive stock by equalizing founder contributions. This minimizes deleterious effects that can arise from inbreeding and genetic drift, which often impact small, isolated populations (Frankham, 2008). Genetic management can also be important when endangered populations are threatened by emerging diseases, which can be particularly devastating for populations that are small and genetically homogeneous (McKnight et al., 2017).

In captive breeding programs, genetic management is encouraged by the Association of Zoos and Aquariums (AZA), an independent accreditation organization that promotes the conservation, education, and animal welfare goals of zoos and aquariums in North America (AZA Board of Directors, 2020). In 2016, The AZA Species Survival Plan Yellow Program, which attempts to retain genetic diversity in endangered species among AZA institutions made a commitment to preserve the genetic diversity of the Eld's deer (Reed et al., 2016).

Eld's deer (*Rucervus eldii*, synonyms include *Cervus eldii* and *Panolia eldii*) are mid-sized deer that are endemic to the tropical forests and wetlands of Southeast Asia (Aung et al., 2001; Thu et al., 2019). The species originally ranged from Northeastern India to Myanmar, Thailand, Cambodia, Laos, and Hainan Island in China (Aung et al., 2001). Currently Eld's deer are found in small remnants of their historic range due to overharvesting and habitat degradation (McShea et al., 1999), and are now listed as endangered by the International Union for the Conservation of Nature and Natural Resources (IUCN) (Gray et al., 2015).

Eld's deer are in the subfamily Cervinae within the family Cervidae. The three subspecies of Eld's deer are *R. e. eldii, R. e. siamensis* and *R. e. thamin* (Thu et al., 2019). The most common subspecies is *R.e. thamin*, which occurs in Myanmar in wild and captive populations (McShea et al., 1999). This subspecies is also managed in captive breeding programs in Eurasian and North American zoos (Thu et al., 2019), including the institutions accredited by the AZA that provided samples for the current study. In 2003, the Cervid Taxon Advisory Group of the AZA, which manages cervids including Eld's deer, released recommendations designed to minimize the threat posed to the cervid stocks of AZA member institutions by the emergence of chronic wasting disease (CWD) (AZA Board of Directors, 2003).

Chronic wasting disease is a transmissible spongiform encephalopathy caused by misfolded prion proteins, which was first detected in mule deer in Colorado in the 1960s and has since spread across wild and captive cervid populations in at least 29 US states and two Canadian provinces, affecting white-tailed deer, mule deer, elk, and moose (Williams and Young, 1980; Williams and Young, 1982; Belay et al., 2004; Baeten et al., 2007; Rivera et al., 2019; Richards, 2021a; Richards, 2021b). CWD has spread to captive cervid populations in South Korea (CDC, 2022; Richards, 2021a; Richards, 2021b) and has also recently been detected in wild cervids in Finland, Norway, and Sweden (Pirisinu et al., 2018; Rivera et al., 2019; Richards, 2021b). Once CWD becomes endemic in a population, it can cause population declines (Haley et al., 2011; Saunders et al., 2012; Edmunds et al., 2016; Davenport et al., 2017).

CWD can be transmitted by direct contact of a susceptible animal with an infected cervid. Infectious prions can be shed by infected animals in semen (Kramm et al., 2020), urine (Haley et al., 2011), saliva, blood (Mathiason et al., 2006), and other bodily fluids (Miller et al., 2004; Gough and Maddison, 2010). CWD is a risk to the management of cervids in captive-breeding programs, even when direct contact with an infected animal is unlikely, because of the possibility of environmental transmission such as through dust inhalation of infectious particles (Miller et al., 2004; Gough and Maddison, 2010) or the infection risk posed by enclosures that previously housed CWD-positive deer (Mathiason et al., 2009). Prions can persist long term in the environment and can remain infectious in soil (depending on the composition of the soil) (Johnson et al., 2006; Kuznetsova et al., 2020) and have been detected in salt licks visited by wild deer (Plummer et al., 2018). Captive cervids such as Eld's deer are at risk due to contaminated feed or bedding (Saunders et al., 2012; Henderson et al., 2015). Another concern is potential transmission during artificial insemination if the sire has infected semen (Kramm et al., 2020).

The prion protein is encoded by the gene *PRNP*. Variation in *PRNP* has been associated with differences in susceptibility to

CWD in cervids. For example, in white-tailed deer (*Odocoileus virginianus*), two non-synonymous single nucleotide polymorphisms (SNPs) have been associated with reduced susceptibility to CWD: c.285A>C that encodes a histidine (H) instead of the more common glutamine (Q) at codon 95 and c.286G>A that encodes a serine (S) instead of the more common glycine (G) at codon 96 (Johnson et al., 2003; O'Rourke et al., 2004; Kelly et al., 2008; Brandt et al., 2015; Brandt et al., 2018). In mule deer (*O. hemionus*) two non-synonymous mutations, at codons 20 from aspartic acid (D) to glycine and 225 from serine to phenylalanine (F) have been identified (Jewell et al., 2005; Wilson et al., 2009; Zink et al., 2020; LaCava et al., 2021). In free-ranging mule deer in Wyoming and Colorado, a phenylalanine encoded by codon 225 is associated with a significantly lower CWD-positive rate compared to mule deer with a serine encoded by codon 225. In western Canada (Wilson et al., 2009) and Nebraska (Zink et al., 2020), a higher proportion of mule deer carrying one aspartic acid and one glycine at codon 20 was detected than mule deer carrying two aspartic acids at codon 20 in CWD-positive mule deer. In orally inoculated mule deer, PrP^{CWD} was detected in the nervous system of deer carrying two serines at codon 225 deer after 189 days, but in deer carrying both a serine and a phenylalanine at codon 225 after 482 days, with the latter showing slower disease progression (Fox et al., 2006). An inoculation study of Rocky Mountain elk (*Cervus canadensis nelsoni*) with either leucine (L) or methionine (M) at codon 132 found that the incubation period of CWD was longest for LL individuals, intermediate for LM, and shortest for MM (Moore et al., 2020). When brain homogenate of CWD-infected elk of various genotypes at this codon was inoculated intracranially into transgenic mice, the incubation periods were found to similarly vary with elk genotype (Moore et al., 2020). In an oral inoculation study, caribou from North America encoding one asparagine (N) and one serine at codon 138 showed resistance to infection from CWD derived from elk and white-tailed deer (Mitchell et al., 2012). However, clinical CWD was detected in caribou with the same polymorphism in an intracranial inoculation study albeit with a reduction in symptomology as compared to the homozygotes (Moore et al., 2016). Fallow deer, in which 138N appears to be fixed (Robinson et al., 2019), showed no evidence of susceptibility to CWD under experimental exposure that mimicked natural transmission (Rhyan et al., 2011) but were found to be susceptible after intracerebral exposure with a prolonged incubation period (Hamir et al., 2011). Thus, when animals are intracerebrally or orally inoculated with variable doses of infectious material, this can lead to the clinical development of CWD even in animals with less susceptible genotypes. However, these experimental conditions may be considered extreme and not typical of susceptibility under natural conditions (Cullingham et al., 2020).

PRNP alleles that have been previously associated with CWD susceptibility can be examined in endangered cervids in captive breeding programs, as an initial step in extrapolating their potential susceptibility to CWD (Perrin-Stowe et al., 2021). In this study, we sequenced *PRNP* in Eld's deer (*R. e. thamin*) housed in AZA-accredited facilities, to determine the degree of polymorphisms in *PRNP*, and to extrapolate the potential susceptibility of Eld's deer to CWD based on the effects of *PRNP* polymorphisms on susceptibility to CWD in other cervid taxa.

Materials and methods

Nomenclature

The taxonomic classification of Eld's deer has been a subject of discussion (Pitra et al., 2004; Heckeberg, 2020; Ghazi et al., 2021; Wong et al., 2021). Some sources assign Eld's deer to the distinct genus *Panolia* following the nomenclature used by John Edward Gray in 1843 (Gray, 1843). In some publications, Eld's deer are placed in the genus *Rucervus*, as they share morphological traits with the other species assigned to the genus, *Rucervus duvaucelii* and *R. schomburgki* (Geist, 1998; Wong et al., 2021). This designation is followed by the Species Survival Commission of the IUCN, as well as the AZA; thus, we use it here. However, some recent molecular studies have included Eld's deer in the genus *Cervus* (Balakrishnan et al., 2003; Angom et al., 2017; Ghazi et al., 2021). The deer examined by the current study were exclusively from the subspecies *R. e. thamin*. When referring to Eld's deer, we mean members of this subspecies, sometimes referred to as the thamin or Burmese brow-antlered deer.

Eld's deer sampling

Blood or tissue samples from 36 Eld's deer individuals were used for this study. The AZA-accredited facilities that provided these samples were the Smithsonian's National Zoo and Conservation Biology Institute (n = 21) in Front Royal, Virginia; the San Diego Zoo Wildlife Alliance (n = 6) in San Diego, California; the Wildlife Conservation Society at the Bronx Zoo (n = 6) in New York, New York; and the Sedgewick County Zoo (n = 3) in Wichita, Kansas (Supplementary Table 1). Samples were collected during routine veterinary care or came from stored blood or tissue collections. This research project was conducted under the Illinois Institutional Animal Care and Use Committee protocol 18212 and the Smithsonian Animal Care and Use Committee protocol #19-13.

DNA amplification and sequence analysis

DNA from tissue samples was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). DNA

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from blood samples was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Germantown, MD). Blood sample lysing incubation time was extended to one hour, while tissue samples were lysed for 24 hours. The manufacturers' instructions for both kits were followed for all other aspects of the protocols. PCR was conducted in 25 µl total volume, containing 1× PCR Buffer II (Applied Biosystems Inc.), final concentrations of 200 µM of each of the dNTPs, 1.5 mM $MgCl_2$, 0.04 units/µl of AmpliTaq Gold DNA Polymerase (Applied Biosystems Inc.) and 0.4 µM of each oligonucleotide primer. The forward primer 223 (5'-acaccctctttattttgcag-3') and the reverse primer 224 (5'-agaagataatgaaaacaggaag-3') were used to amplify and sequence 830 bp encompassing the complete coding region within exon 3 of *PRNP*. Primer 223 is designed to amplify the functional *PRNP* gene by targeting introns, to avoid a processed pseudogene, which lacks introns, that has previously been detected in cervid taxa (O'Rourke et al., 2004).

The PCR cycling algorithm for *PRNP* amplification was as follows: initial denaturing at 95°C for 10 mins; 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min (5 cycles); 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min (40 cycles); and a final extension at 72°C for 7 min (Perrin-Stowe et al., 2020). PCR amplification was confirmed on a 1.0% agarose gel with ethidium bromide using gel electrophoresis. The successful amplification products were enzyme-purified with Exonuclease I (New England Biolabs) and shrimp alkaline phosphatase (New England Biolabs) (Hanke and Wink, 1994). Purified PCR product (1 µl) and a primer (0.12 µM) were used for Sanger sequencing, in both directions, using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI).

In addition to each of the PCR primers, internal primers *PRNP*-IF 5'-atgctgggaagtgccatga-3' and *PRNP*-IR 5'-catggcattcccagcat-3' were also used to sequence the gene (Ishida et al., 2020). These sequences were then resolved on an ABI 3730XL DNA Sequencer at the Keck Center for Functional and Comparative Genomics at the University of Illinois at Urbana-Champaign. Sequences were then visually examined and assembled using the software Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI).

DNA sequence analysis

The software package DnaSP utilizing the algorithm Phase was used to infer haplotypes (Stephens et al., 2001; Librado and Rozas, 2009); 10,000 iterations were run with 1000 burn-in iterations. Gene and haplotype identity was verified using NCBI Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Haplotype sequences were aligned using Sequencher, the open reading frames were confirmed, and the sequences were translated using MEGA X v.10.1 (Kumar et al., 2018). The distinct haplotype sequences were deposited in GenBank (accession numbers: OL961483-OL961485). The software PopART was used to generate and illustrate median-joining networks (under default parameters) (Bandelt et al., 1999; Leigh and Bryant, 2015). Haplotype and nucleotide diversity were calculated using DnaSP (Librado and Rozas, 2009). Confidence intervals for the haplotype frequencies (Hazra, 2017) were calculated using the following equation: $\hat{p} \pm z\sqrt{\frac{\hat{p}(1-\hat{p})}{n}}$

Results

The complete coding region of *PRNP* was successfully sequenced in all 36 Eld's deer individuals. Nucleotide diversity (π) was 0.00402. Out of the seven SNPs identified, five were synonymous: c.63G>C, c.114G>A, c.321G>A, c.516C>T and c.651T>C (Table 1). One of the two non-synonymous SNPs was c.624G>A, which encodes isoleucine (I) instead of methionine (M) at codon 208; 208I has been previously reported in two other cervid species (Table 2). The second non-synonymous SNP was c.676C>G, which encodes glutamic acid (E) instead of glutamine (Q) at codon 226; 226E has been previously reported in several cervid species (Jeong et al., 2007; Haley et al., 2017; Robinson et al., 2019) (Table 2). Each SNP identified (whether synonymous or nonsynonymous) was found in at least 22 chromosomes out of the 72 total chromosomes in the deer assessed.

Three haplotypes were inferred after the sequences were phased. Haplotypes were designated Ret1 through Ret3, in order of frequency. None of these three haplotype sequences has been previously reported among cervid sequences in Genbank. Haplotype diversity (*Hd*) in the Eld's deer samples was 0.636. The haplotype with the highest frequency was used as the reference sequence for the Eld's deer.

Haplotype Ret1 was detected in 35 of 72 (0.486 ± 0.115 [95% confidence interval: 95% CI]) phased Eld's deer sequences and had the highest frequency among the samples (Table 1). The frequencies and 95% confidence intervals for each of the haplotypes are shown in Table 1, as are the SNPs present in each of the haplotypes. The three haplotypes encoded two different prion protein (PrP) variants. Haplotypes Ret1 and Ret3 encoded the same amino acid sequence (Table 2). An amino acid sequence identical to that encoded by Ret1 and Ret3 has been previously reported from at least one individual in a number of other cervid species: white-tailed deer (GenBank accession number: MG856905), Rocky Mountain mule deer (*Odocoileus hemionus hemionus*) (AAC33174), European roe deer (*Capreolus capreolus*) (MK103016), sika deer (MK103018), Reeves's muntjac (*Muntiacus reevesi*) (MK103020), and Chinese water deer (MK103024) (Table 2). The other haplotype, Ret2, encodes an amino acid sequence identical to that encoded by haplotype Elad2 in Pere David's deer (GenBank accession number: MW804583) (Perrin-Stowe et al., 2021). Haplotype Ret2 encodes an isoleucine (I) at codon 208 and glutamic acid (E) at codon 226. This variant will be referred to as PrP variant

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TABLE 1 *PRNP* SNPs in Eld's deer haplotypes.

Haplotype	Nucleotide position in the coding region								95% CI
	63	114	321	516	624	651	676	*n*	
Ret1	G	G	G	C	G	T	C	35	0.486 ± 0.115
Ret2	C	A	A	T	**A**	C	**G**	22	0.306 ± 0.106
Ret3	.	.	A	.	.	.	.	15	0.208 ± 0.094

Single nucleotide polymorphisms (SNPs) within the prion protein gene *PRNP* are compared across Eld's deer (*Rucervus eldii thamin*) haplotypes sequenced for this study. Haplotypes were numbered in order of frequency. Nucleotides matching those in haplotype Ret1 are shown as dots, while the character state is shown for those that differ. Nucleotidesin boldface indicate non-synonymous SNPs relative to haplotype Ret1. Guanine at position 624 (codon 208) and cytosine at position 676 (codon 226) encode methionine and glutamine, respectively. Adenine at position 624 (codon 208) and guanine at position 676 (codon 226) encode isoleucine and glutamic acid, respectively. CI is an abbreviation for confidence interval; *n* is the number of chromosomes carrying each haplotype.

208I;226E, while PrP variant encoded by Ret1 and Re3 will be referred to as PrP variant 208M;226Q.

A median-joining network of the three Eld's deer haplotype sequences is shown in Figure 1. The differing amino acids encoded by the haplotypes are also shown. Ret1 and Ret3 are different at a single nucleotide, and thus more similar to each other than to Ret2 (separated from Ret1 and Ret3 by at least six nucleotides). The Eld's deer haplotypes were also compared to *PRNP* sequences of other cervid taxa that had available sequences on GenBank and encoded variants of PrP (Figure 2, Table 2). Some Eld's deer haplotypes are more similar to *PRNP* sequences in other cervid species than they are to each other. Haplotypes Ret1 and Ret3 are more similar to haplotypes of the Reeve's muntjac and the Rocky Mountain mule deer than they are to haplotype Ret2 (Figure 2). Haplotype Ret2 is more similar to haplotypes carried by the Iberian red deer (*C. elaphus hispanicus*), the sika deer (with the 226E substitution), Rocky Mountain elk, and Pere David's deer haplotype Elad2 (Figure 2).

Discussion

In the *PRNP* coding region of 36 Eld's deer individuals, seven polymorphisms were identified, comprising three haplotypes. The three haplotypes encoded two different PrP protein variants. One amino acid difference between the PrP variants was a methionine (M) to isoleucine (I) substitution at codon 208. This is a conservative amino acid substitution with both amino acids being relatively unreactive and hydrophobic (Betts and Russell, 2003). This substitution generally does not lead to considerable change in structure or function to a protein (Ohmura et al., 2001). However, in a recombinant protein misfolding cyclic amplification (PMCA) study, the M208I substitution has been reported to inhibit propagation of prions across the transmission barrier between deer and sheep (Harrathi et al., 2019). This finding may tentatively suggest that cervids that carry PrP with 208I may be less susceptible to CWD than cervids that carry PrP with 208M (Harrathi et al., 2019). However, that study was conducted *in vitro* using PMCA and thus this hypothesis would need to be tested, e.g., *via* additional inoculation experiments *in vivo* using brain homogenate from cervids with the relevant polymorphisms or in transgenic mice expressing cervid PrP.

The other polymorphism that distinguishes the Eld's deer PrP variants is a glutamine (Q) to glutamic acid (E) amino acid substitution at codon 226. This is a conservative substitution; both amino acids are polar and have similar physiochemical characteristics (Betts and Russell, 2003). Transgenic mice expressing PrP with 226E (carried by Rocky Mountain elk and other cervid species) developed CWD through experimental inoculation more quickly than transgenic mice expressing PrP with 226Q (carried by mule deer and white-tailed deer) (Angers et al., 2009; Angers et al., 2010). The mice with the 226Q substitution showed a slower incubation rate but ultimately still experienced CWD infection (Angers et al., 2010; Bian et al., 2019). Red deer (*Cervus elaphus elaphus*) may encode either variant at codon 226 (Balachandran et al., 2010). A study of four inoculated red deer showed no difference in CWD incubation time between homozygotes for 226E or 226Q when compared to the heterozygote 226E;226Q (Balachandran et al., 2010). Depending on the CWD strains and cervid species, the residues at 226 may have a differential effect on CWD prion propagation efficiency, but neither of these residues seems to completely remove susceptibility to CWD. Further investigation into both non-synonymous substitutions within Eld's deer specifically would be necessary to determine their potential role in CWD infection in this species.

Various other cervid species carry *PRNP* that encodes both 208M and 226Q, which are encoded by the Eld's deer haplotypes Ret1 and Ret3 (PrP 208M;226Q) (Table 2). The Eld's deer PrP 208M;226Q has the same amino acid sequence as the PrP variant found most frequently in white-tailed deer (Table 2); this PrP variant is disproportionately common among white-tailed deer positive for CWD (Ishida et al., 2020). Thus, management of Eld's deer in captive breeding facilities may consider increasing the frequency of PrP variant 208I;226E, because Eld's deer carrying PrP variant 208M;226Q may be at a greater risk for CWD transmission from free-ranging white-tailed deer, which are the cervids with the largest distribution among deer in North America (Hewitt, 2015). Free-ranging mule deer also carry this

TABLE 2 PrP variation in various cervid taxa.

Cervid Subfamily	Taxon	Common Name	Designation	Codons											Source
				95	96	98	109	132	138	176	208	209	225	226	
Cervinae	*Rucervus eldii thamin*	Eld's deer	Ret1	Q	G	T	K	M	S	N	M	M	S	**Q**	This study
	Rucervus eldii thamin	Eld's deer	Ret2	.	.	.	.	.	.	.	**I***	.	.	E	This study
	Rucervus eldii thamin	Eld's deer	Ret3	.	.	.	.	.	.	.	.	.	.	.	This study
	Elaphurus davidianus	Pere David's deer	Elad1	.	.	.	.	.	N	.	.	.	.	.	KC476497
	Elaphurus davidianus	Pere David's deer	Elad2	.	.	.	.	.	.	.	I	.	.	E	MW804583
	Cervus nippon	Sika deer		.	.	.	.	.	.	.	.	.	.	E	AY679695
	Cervus nippon	Sika deer	Haplotype 1	.	.	.	.	.	.	.	.	.	.	.	MK103018
	Dama dama	Fallow deer		.	.	.	.	.	N	.	.	.	.	E	MK103017
	Cervus elaphus	Red deer		–	–	–	–	–	.	–	–	–	–	.	Robinson et al., 2019
	Cervus elaphus	Red deer		–	–	–	–	–	–	–	I	–	–	–	Kaluz et al., 1997
	Cervus elaphus hispanicus	Iberian red deer	226E	.	.	.	.	.	.	.	.	.	.	E	KT845864
	Cervus elaphus nelsoni	Rocky Mountain elk		.	.	.	.	.	.	.	.	.	.	E	EU082291
	Cervus elaphus nelsoni	Rocky Mountain elk	L132	.	.	.	.	L	.	.	.	.	.	E	AF016228
	Muntiacus reevesi	Reeves's muntjac		.	.	.	.	.	.	.	.	.	.	.	MK103020
Capreolinae	*Odocoileus virginianus*	White-tailed deer	PrP variant A	.	.	.	.	.	.	.	.	.	.	.	MG856905
	Odocoileus virginianus	White-tailed deer	PrP variant C	.	S	.	.	.	.	.	.	.	.	.	MG856907
	Odocoileus virginianus	White-tailed deer	PrP variant F	H	.	.	.	.	.	.	.	.	.	.	MG856910
	Odocoileus virginianus	White-tailed deer		–	–	–	–	–	–	–	–	–	–	.	Angers et al., 2010
	Odocoileus hemionus	Mule deer		–	–	–	–	–	–	–	–	–	F	–	Jewell et al., 2005
	Odocoileus hemionus	Mule deer		–	–	–	–	–	–	–	–	–	–	.	Angers et al., 2010
	Odocoileus hemionus hemionus	Rocky Mountain mule deer		.	.	.	.	.	.	.	.	.	.	.	AAC33174
	Rangifer tarandus tarandus	Montain reindeer (Caribou)		.	.	.	.	.	.	D	.	.	.	.	MK097270
	Rangifer tarandus tarandus	Montain reindeer (Caribou)		–	–	–	–	–	N	–	–	–	–	–	Cheng et al., 2017
	Alces alces	Moose		.	.	.	Q	.	.	.	.	.	.	.	JQ290077
	Alces alces shirasi	Shiras moose		.	.	.	.	.	.	.	.	I	.	.	AY225485
	Capreolus capreolus	European roe deer		.	.	.	.	.	.	.	.	.	.	.	MK103016
	Hydropotes inermis	Chinese water deer		.	.	.	.	.	.	.	.	.	.	.	MK103024

PrP (prion protein) amino acid sequences from various cervids compared with the variation present in the Eld's deer (*Rucervus eldii thamin*) samples sequenced for this study. The translation of Ret1, the most common haplotype among Eld's deer, is used as a reference. Amino acids that match the reference are denoted by a period, while variations are shown as amino acid abbreviations. Dashes indicate missing information. Note that both of the amino acid sequences in Eld's deer match those of other species. Amino acids associated with reduced vulnerability to CWD in a taxon are outlined. The cervid taxa for which references are listed did not have a complete sequence available within the NCBI GenBank database but reported alleles in cervids associated with varying vulnerability to CWD and therefore were included in the table. Amino acids that have been associated with reduced vulnerability of CWD in other taxa and are carried by the Eld's deer are in boldface. Asterisk indicates that this amino acid found in Eld's deer has been previously reported to be associated with reduced vulnerability to CWD in sheep and limits the propagation of CWD to cervids. Protein variant designations for white-tailed deer follow those of Ishida et al., 2020; other designations may refer to a haplotype, SNP or PrP variant for the taxon listed.

PrP variant (Table 2), and thus could also be a source of infection. This concern is notable given that interspecies transmission of CWD is common among cervids (Saunders et al., 2012) and that the AZA has already flagged transmission from free-ranging cervids as a potential threat to cervids in their facilities (AZA Board of Directors, 2020). Increasing the frequency of PrP 208I;226E would be preferred given that it differs in amino acid sequence from PrP in North American cervids. While other genetic management goals (such as equalizing founder contributions and avoidance of

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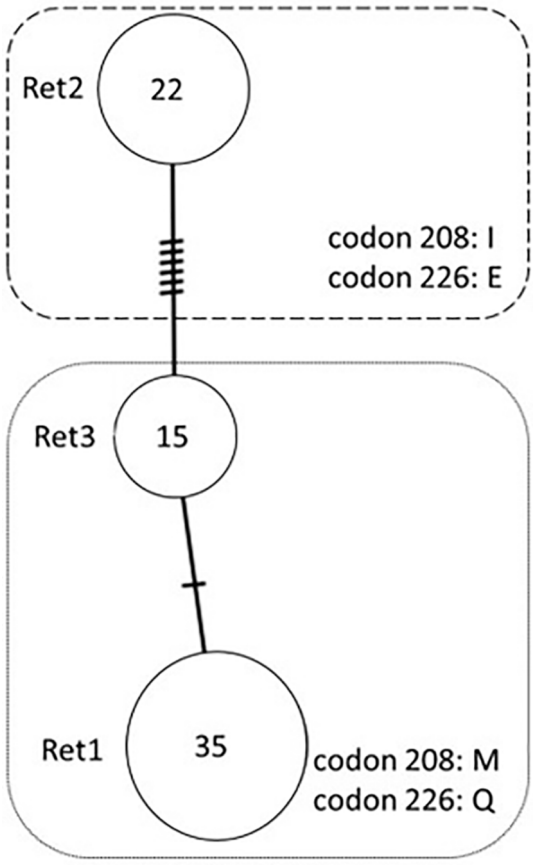

FIGURE 1
Median-joining network of *PRNP* haplotypes in Eld's deer (*Rucervus eldii thamin*). Each circle represents a distinct haplotype; each hatch mark on the branches separating circles represents a mutation. The size of each circle is proportional to the number of chromosomes carrying the haplotype, which is also listed within the circle. The designation for each haplotype is listed beside the circle. There were two non-synonymous mutations, for which encoded amino acids are shown within the dotted boxes. The box outlined by small dots includes the two haplotypes that encode PrP variant 208M;226Q (Ret1 and Ret3). The box outlined by dashes includes the single haplotype that encodes PrP variant 208I;226E (Ret2).

inbreeding) must be prioritized, the susceptibility of Eld's deer to CWD infection could potentially be reduced by increasing the frequency of haplotypes encoding 208I;226E.

Trans-species polymorphisms, multiple alleles shared by more than one species, can be an indication of long-term balancing selection (Klein et al., 1998; Charlesworth, 2006; Koenig et al., 2019). Trans-species polymorphisms were evident when Eld's deer *PRNP* sequences were compared to those of other cervids (Figure 2). In Eld's deer, some *PRNP* haplotypes are less similar to each other than to *PRNP* sequences in other cervid taxa (Figure 2), providing support for this hypothesis. The two Eld's deer PrP variants may have been present in a common ancestor of some species within the subfamily Cervinae (Klein et al., 1998; Charlesworth, 2006; Koenig et al., 2019), and persist within modern Eld's deer. A similar pattern is shown when Pere David's deer *PRNP* haplotypes are compared to the *PRNP* sequences of other cervid species (Perrin-Stowe et al., 2021). While there have been no reports of wild cervid taxa within Asia that have been exposed to the recent outbreak of CWD (we note that wider assessments are likely needed), historical exposure of ancestral populations to prion diseases driving balancing selection cannot be ruled out. There is evidence for balancing selection affecting *PRNP* in response to historical epidemics of transmissible spongiform encephalopathies caused by prions such as scrapie in sheep and kuru in humans (Mead et al., 2003; Slate, 2005; Nyström and Hammarström, 2014). Heterozygote advantage is believed to play a role in kuru in humans, with lower disease susceptibility for those encoding at codon 192 of the prion gene methionine in one chromosome and valine in the other chromosome (Nyström and Hammarström, 2014).

CWD susceptibility and progression can differ in incubation time and neuropathology due to variation both in prion strains and in PrP (Bruce et al., 1994; Bruce, 2003; Collinge and Clarke, 2007; Angers et al., 2014; Moore et al., 2020). This suggests that the presence of multiple PrP variants in a population could confer possible fitness benefits, as one variant may be more or less susceptible to a certain CWD strain than another. As noted, PrP variant 208M;226Q in Eld's deer is identical to the most common PrP sequence carried by white-tailed deer and is also present in mule deer. This may potentially cause Eld's deer that carry *PRNP* encoding the same PrP as wild North American cervids to be at greater risk of inter-species transmission of CWD than Eld's deer with a different PrP variant. At the same time, deer that carry PrP variant 208I;226E might also be susceptible to certain prion strains because transgenic mice that carry 226E (which is carried by Rocky mountain elk among other species) develop disease faster than those that carry 226Q (carried by white-tailed deer, mule deer, and various other species) (Table 2) (Angers et al., 2010; Bian et al., 2019). Despite a potential risk, the potential advantage to deer that carry isoleucine (I) at position 208, and the benefits of maintaining more than one PrP variant in a population suggest that retention of both protein variants should be a goal in the management of Eld's deer populations. In white-tailed deer, reduced susceptibility to CWD is provided to deer that are heterozygous and carry a single copy of a protective haplotype (Brandt et al., 2018; Ishida et al., 2020). This may suggest that the Ret1 and Ret3 haplotypes could be maintained in the stock in a heterozygous state with Ret2.

The interconnected population of Eld's deer within AZA-accredited facilities are descended from 15 founders. The founder genome equivalent is 4.29, meaning that approximately four unrelated deer would have similar genetic diversity to the population managed in the captive breeding program (Reed et al., 2016). The AZA Species Survival Plan Yellow Program for Eld's

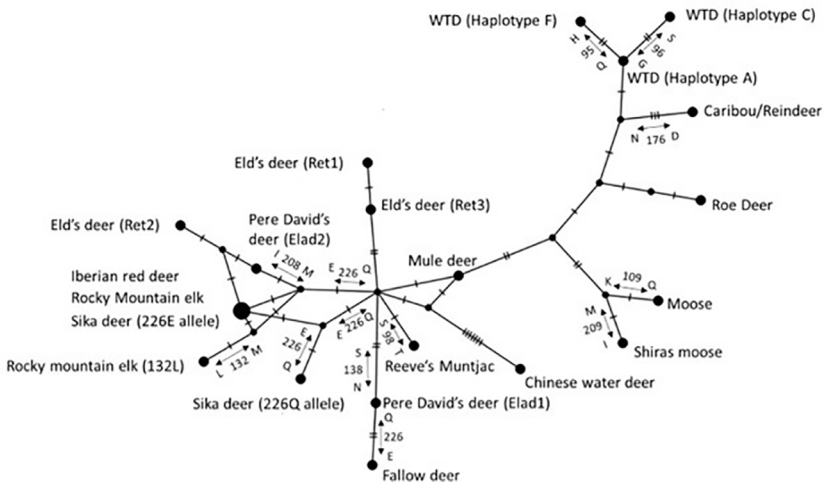

FIGURE 2
Median-joining network showing variation among *PRNP* sequences in cervid taxa. Each labeled circle represents a distinct haplotype and each hatch mark on the branches represents a single nucleotide substitution. For non-synonymous substitutions, the amino acid abbreviations are listed along the branches with their codon number. Eld's deer haplotypes Ret1 and Ret3 group together, while haplotype Ret2 is more similar to sequences from other cervids than to haplotypes Ret1 and Ret3. WTD is the abbreviation for white-tailed deer. From three of the species shown, identical *PRNP* sequences have been reported: Iberian red deer (KT845864), sika deer (those with the 226E substitution; AY679695), and Rocky Mountain elk (EU082291). The node that represents the *PRNP* sequence from these three species is drawn larger than the nodes that represent the *PRNP* sequence from a single species.

deer indicates that some founder lineages might be underrepresented within Eld's deer (Reed et al., 2016). Despite this, the retention of three haplotypes of *PRNP* that encode two PrP variants in Eld's deer would point to considerable success for the captive breeding program in maintaining the genetic diversity of the Eld's deer stock. Furthermore, ex situ management of Eld's deer within AZA facilities has included mandatory testing for CWD of all animals more than 6 months old that die (Spencer, 2008). Thanks to precautions (fencing, limiting interactions with wild cervids) taken by animal managers, none of the Eld's deer to date have tested positive for CWD, demonstrating the importance of good animal husbandry practices in CWD prevention in ex situ managed Eld's deer populations.

To our knowledge, this study is the first to report *PRNP* sequences and to identify *PRNP* polymorphisms in the Eld's deer. Because Eld's deer are endangered, and experimental methods can be invasive and costly, CWD inoculation experiments to directly determine their actual degree of susceptibility to CWD infection may be difficult to conduct or inappropriate. Therefore, studies that investigate the potential genetic susceptibility to CWD in Eld's deer by comparing their PrP to that of other deer species that have the same PrP variants may provide information useful for managing the species to minimize the risk of CWD. Strict management practices that focus on avoiding contact between non-native cervid species and susceptible North American cervids (including contact with potentially contaminated feed, bedding and enclosures) are necessary to slow the spread of CWD. Additionally, an important management recommendation for Eld's deer specifically is to lower the frequency of the Ret1 and Ret3 haplotypes encoding the PrP variant that is identical in amino acid sequence to a PrP variant that is common in North American deer and is disproportionately common among white-tailed deer with CWD.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material. PRNP haplotypes of Eld's deer generated from this study have been deposited in GenBank under the accession numbers OL961483-OL961485.

Ethics statement

The animal study was reviewed and approved by Illinois Institutional Animal Care and Use Committee protocol 18212 and the Smithsonian Animal Care and Use Committee protocol #19-13.

Author contributions

TP-S generated data, conducted data analysis, and co-wrote the manuscript. YI assisted in data analysis and co-wrote the manuscript. DR and OR assisted in sample collection and

contributed to interpretation and writing of the manuscript. ET assisted in sample collection and data generation. BP assisted in sample collection and the framing and writing of the manuscript. JN and NM-P contributed to the interpretation and writing of the manuscript. AR was responsible for the initiation of the study, helped manage the study and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcosc.2022.1007100/full#supplementary-material

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