



Variation in the *PRNP* gene of Pere David's deer (*Elaphurus davidianus*) may impact genetic vulnerability to chronic wasting disease

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Abstract

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy caused by prions that has spread across cervid species in North America since the 1960s and has recently been detected in Eurasian cervids. The Association of Zoos and Aquariums (AZA) considers CWD to be of major concern for cervids in AZA-accredited facilities because of the indirect transmission risk of the disease and the impact of CWD regulatory protocols on captive breeding programs. Vulnerability to CWD is affected by variation in the *PRNP* gene that encodes the prion protein. We therefore sequenced *PRNP* in Pere David's deer (*Elaphurus davidianus*), a species that was extinct in the wild for more than a century and descends from ca. 11 founders. In 27 individuals, we detected two *PRNP* haplotypes, designated Elad1 (51 of 54 sequences) and Elad2 (3 of 54 sequences). The two haplotypes are separated by four single nucleotide polymorphisms (SNPs), three of which are non-synonymous. Both Elad1 and Elad2 have polymorphisms that in other cervid taxa are associated with reduced vulnerability to CWD. The two haplotypes are more similar in sequence to *PRNP* in other cervids than to each other. This suggests that *PRNP* in cervids may have been under long-term balancing selection, as has been shown for *PRNP* in non-cervid taxa, and which could account for the presence of multiple haplotypes among founders. There may be a fitness benefit in maintaining both *PRNP* haplotypes in the species because variation in the prion protein amino acid sequence can limit transmission of CWD.

Keywords Cervids · Encephalopathy · Milu · Transmissible spongiform encephalopathy

Introduction

Chronic wasting disease (CWD) is a highly transmissible spongiform encephalopathy (TSE) caused by prions (Williams and Young 1980; Belay et al. 2004). Prions are “proteinaceous infectious particles” that arise when a normal cellular prion protein (PrP^C) misfolds into a protein with an abnormal yet stable protein structure (Prusiner 1982, 1991; Belay et al. 2004). This abnormal structure (PrP^{CWD}) causes the cellular prion protein to become infectious, binding to a normal PrP^C to induce conformational changes to render it an abnormal PrP^{CWD}. Prions build up over time, and cause the deterioration of the organism's nervous system due to the accumulation of the insoluble and toxic PrP^{CWD} aggregates. This accumulation leads to cell death, and the sponge-like damage to brain tissue that characterizes transmissible spongiform encephalopathies (TSEs). CWD infection is invariably fatal, with no known treatment or cure (Williams and Young 1980; Belay et al. 2004). CWD can be spread via direct animal to animal contact (infectious to a susceptible

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animal) through exchange of bodily fluids such as saliva, urine, blood, and semen (Mathiason et al. 2006; Haley et al. 2009, 2011; Kramm et al. 2020), although direct contact with wild cervids is a less likely occurrence for deer kept in most zoos than for deer kept in deer ranches. Prions are also sequestered by plants and remain infectious in certain types of soil particles for years (Nichols et al. 2009; Bartelt-Hunt and Bartz 2013; Kuznetsova et al. 2014, 2018). They can also remain infectious on a variety of materials, including glass, plastics, and steel instruments even after autoclaving (Edgeworth et al. 2009; Pritzkow et al. 2018). Environmental CWD transmission to mule deer from paddocks that previously held infected animals has been experimentally confirmed as well (Miller et al. 2004). Epidemiological models indicate that the indirect transmission risk of contracting CWD may be substantial (Almberg et al. 2011; Saunders et al. 2012).

CWD was initially characterized in the 1960s in Colorado and has since spread geographically in North America across populations of both free-ranging and captive cervids in at least 26 U.S. states and three Canadian provinces (Williams and Young 1980, 1982; Rivera et al. 2019; CDC 2020; Richards 2021). U.S. federal and state agencies (including the U.S. Fish and Wildlife Service) actively manage, monitor, and are attempting to reduce the spread of CWD (Mateus-Pinilla et al. 2013; Manjerovic et al. 2014). Despite these efforts, CWD has recently spread to captive populations in South Korea and was detected in wild cervid populations in Finland, Norway and Sweden (Richards 2021). Global concern for CWD containment has increased because CWD may cause population declines after becoming endemic in a cervid population (Edmunds et al. 2016).

The increasing geographic spread of CWD has raised worries about its potential to impact the health of endangered cervids in the wild or captive breeding programs (CDC 2020). Small populations such as those kept in captive breeding programs are particularly vulnerable to the impact of infectious disease (Viggers et al. 1993; Cunningham et al. 2017). The Association of Zoos and Aquariums (AZA) has established guidelines for the monitoring of CWD, and for the management of cervids that could be vulnerable to CWD (AZA Board of Directors 2003). However, CWD can and has been transmitted to captive populations via infected feed and bedding (fomites) and not just from direct transmission by infected animals (Mathiason et al. 2006, 2009; Henderson et al. 2015) indicating that cervids in AZA-accredited institutions could be at risk of transmission from infected animals and fomites. CWD has also been detected in cervid semen, raising concerns on how this may impact the use of artificial insemination in captive cervid facilities (Kramm et al. 2020).

Another interest of AZA-accredited institutions is to better understand the risk of CWD transmission to determine

if current protocols regarding the transport of cervids are appropriate. Strict restrictions on interstate transfers can hinder cervid breeding programs in AZA-accredited facilities. Thus, further elucidating transmission risk is important so that more precise protocols and restrictions can be established. The AZA Cervid Taxon Advisory Group monitors cervid species in captive breeding programs, including the Pere David's deer (*Elaphurus davidianus*), which has been categorized by the IUCN red list (<https://www.iucnredlist.org>) as extinct in the wild.

The deer family Cervidae is divided into the subfamilies Cervinae and Capreolinae (Heckeberg 2020). The subfamily Capreolinae includes the cervid species of North and South America, and some species found in Eurasia (Fernández and Vrba 2005). Pere David's deer is within the subfamily Cervinae, which also includes the red deer (*Cervus elaphus*), elk or wapiti (*Cervus canadensis*), sika deer (*Cervus nippon*), Eld's deer (*Panolia eldii*), fallow deer (*Dama dama*), spotted deer (*Axis axis*), and barasingha (*Rucervus duvaucelii*), among others. This subfamily originated in Eurasia and is characterized by elaborate antlers at obtuse angles and shared plesiometacarpal traits (Pitra et al. 2004).

Pere David's deer (also called the milu) historically ranged across the swamplands of eastern Asia with the largest populations found in southern mainland China. There are accounts of the species being kept in captivity dating back over a thousand years (Schafer 1968). Pere David's deer became extinct in the wild in the late nineteenth century due to overhunting (Schafer 1968; Zhu et al. 2018; Yuan et al. 2019). The last known population within China was kept in the Nanyang Royal Hunting Garden; these deer were killed during the Boxer Rebellion of 1899–1901 (Schafer 1968; Jiang et al. 2000; Li et al. 2011). The modern population is descended from a herd of 18 deer (11 of which are believed to have left descendants) housed in the United Kingdom at Woburn Abbey during the early twentieth century (Jones 1951; Zeng et al. 2007; Turvey et al. 2017; Zhang et al. 2017).

In the 1980s, Pere David's deer began to be reintroduced as free-ranging herds in China, with the largest reintroduced population now residing in the Dafeng coastal region; this population grew from 39 individuals reintroduced in 1986 to over 5,000 individuals in 2020 (Jiang et al. 2000; Hu and Jiang 2002; Zhu et al. 2018; Zhang et al. 2021). However, much of the population still resides in captive facilities, which are used to supplement the free-ranging populations in China (Zhu et al. 2018). Despite being descended from only 11 individuals, Pere David's deer have not exhibited obvious adverse effects indicative of inbreeding depression (Yuan et al. 2019). Deleterious alleles may have been purged during the continuous population decline (Zhu et al. 2018). Also, despite the low number of founders, Pere David's deer do not seem to exhibit negative genetic effects, or the

extreme lack of genetic diversity expected of a population that undergoes a severe bottleneck (Zhang et al. 2016; Zhu et al. 2018). Pere David's deer are often mentioned as a model of successful captive population management since the population has rebounded from so few founders (Jiang et al. 2000; Zhu et al. 2018).

As with other cervids, there is concern that Pere David's deer could be susceptible to CWD. CWD has not yet been reported in counties where AZA-accredited institutions maintain Pere David's deer in captive breeding programs (Supplementary Fig. 1). However, cervids in these facilities are still at risk because it is possible that CWD can be transmitted through feed and bedding, and direct transmission from cervids brought in from non-accredited captive settings or potential infection from semen during artificial insemination (Mathiason et al. 2009; Henderson et al. 2015; Kramm et al. 2020).

The gene *PRNP* encodes the prion protein. Genetic variation in *PRNP* has been associated with substantial differences in vulnerability to CWD in many cervid taxa. For example, there is evidence from fallow deer and caribou that asparagine at codon 138 is associated with reduced vulnerability to CWD (Rhyan et al. 2011; Mitchell et al. 2012; Moore et al. 2016; Cheng et al. 2017; Haley et al. 2017). Variation at codons in other taxa has also been associated with differences in CWD vulnerability. These differences may be due to full or partial resistance to natural transmission of the disease, to delayed onset of infection, unstable prion propagation or varying resistance to different CWD strains (Béringue et al. 2008; Rhyan et al. 2011). In some cases, intracerebral inoculation can lead to disease transmission (if sometimes delayed or incomplete) even if direct or secondary transmission of CWD has not been experimentally shown or detected in nature (Hazards et al. 2017; Arifin et al. 2021).

While *PRNP* has been sequenced in two Pere David's individuals (KC476497 and PRJNA256236), the degree and type of variation has not been broadly examined. Therefore, we here sequenced the entire coding region of *PRNP* in 27 Pere David's deer, to inform the conservation and management of the species by examining *PRNP* variation. Despite the limited number of founders, we detected *PRNP* variation in Pere David's deer, including non-synonymous substitutions that in other deer species are associated with reduced vulnerability to CWD.

Materials and methods

Pere David's deer sampling

A total of 27 samples from different Pere David's deer individuals were provided by AZA-accredited institutions

and sequenced for this study. The Wilds, a non-profit safari park and conservation center, located in Cumberland, Ohio, provided 15 samples; the San Diego Zoo Institute for Conservation Research provided six samples; and the Wildlife Conservation Society provided six samples from the Bronx Zoo (Table 1). The samples were from previously stored blood collections or were collected during routine veterinary procedures.

DNA amplification and sequence analysis

DNA was extracted from blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Germantown, MD) using a one-hour lysis time. Manufacturer's instructions were followed in all other aspects of the protocol. The DNA was amplified by PCR in 25 µl total volume, containing 1× PCR Buffer II (Applied Biosystems Inc. [ABI]), final concentrations of 200 µM of each of the dNTPs, 1.5 mM MgCl₂, 0.04 units/µl of AmpliTaq Gold DNA Polymerase (ABI) 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 an 830 bp region encompassing the full open reading frame, encoded by exon 3 of *PRNP* (O'Rourke et al. 2004). Primers 223 and 224 had been designed to avoid amplifying the *PRNP* pseudogene that lacks introns (O'Rourke et al. 2004). PCR conditions for *PRNP* amplification were as follows: 95 °C for 10 min for the initial denaturing; 5 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; and 40 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min; with a final extension of 7 min at 72 °C. PCR amplification was confirmed with a 1.0% agarose gel using gel electrophoresis. The successful amplification products were enzyme-purified (Hanke and Wink 1994). Sequencing was conducted in both directions, relying on each of the PCR primers, with primers *PRNP*-IF 5'-ATGCTGGGAAGTGCCATGA-3' and *PRNP*-IR 5'-CATGGCATTCCCAGCAT-3' used as internal primers (Ishida et al. 2020). Sequences were generated using the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI), with 1.0 µl of purified PCR product and 0.12 µM of primer, 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 confirmed and concatenated using Sequencher software 5.4.6 (Gene Codes Corporation, Ann Arbor, MI).

Haplotype analysis

Phase v.2 within the software DnaSP was used to phase the sequence data and infer haplotypes (Stephens et al. 2001; Librado and Rozas 2009); 10,000 iterations were run with 1000 burn-in iterations. Haplotype sequences were aligned using

Table 1 Pere David's deer sample information

Lab ID	Original ID	Studbook No	Sex	Sample source	Haplotypes	Sire	Dam	Half siblings
EDA1	White 77	313	Male	The Wilds	Elad1	MULT55	238	EDA4
EDA2	White 27	None	Female	The Wilds	Elad1			
EDA3	White 9	228	Male	The Wilds	Elad1	MULT79	206	EDA5, EDA8
EDA4	White 22	312	Male	The Wilds	Elad1	MULT55	209	EDA1, EDA9, EDA12
EDA5	Red 189	221	Female	The Wilds	Elad1	MULT40	206	EDA3, EDA10, EDA12
EDA6	White 29	254	Female	The Wilds	Elad1	MULT4	MULT24	
EDA7	Red 55	250	Male	The Wilds	Elad1	MULT50	239	EDA9, EDA13
EDA8	White 15	226	Male	The Wilds	Elad1	MULT79	MULT52	EDA3
EDA9	Red 53	248	Male	The Wilds	Elad1	MULT50	209	EDA4, EDA7, EDA12, EDA13
EDA10	Red 11	224	Female	The Wilds	Elad1	MULT40	MULT51	EDA5, EDA12
EDA11	White 28	209	Female	The Wilds	Elad1	379	253	
EDA12	White 13	230	Female	The Wilds	Elad1	MULT40	209	EDA4, EDA5, EDA9, EDA10
EDA13	Red 39	310	Male	The Wilds	Elad1	MULT50	MULT57	EDA7, EDA9
EDA14	White 32	239	Female	The Wilds	Elad1	MULT41	MULT42	
EDA15	Green 125	None	Female	The Wilds	Elad1			
EDA16	N2015-0302	290	Male	WCS/Bronx Zoo	Elad1	MULT10	418	
EDA17	N2013-0315	659	Male	WCS/Bronx Zoo	Elad1	290	293	
EDA18	N2014-0422	413	Female	WCS/Bronx Zoo	Elad1	400	410	EDA19, EDA21
EDA19	N2014-0605	401	Female	WCS/Bronx Zoo	Elad1	400	MULT39	EDA18, EDA21
EDA20	N2009-0639	337	Male	WCS/Bronx Zoo	Elad1	MULT1	MULT18	
EDA21	N2013-0728	415	Female	WCS/Bronx Zoo	Elad1	400	414	EDA18, EDA19
EDA22	3732	100,443 (L)	Male	San Diego Zoo	Elad1			
EDA23	3741	101,724 (L)	Female	San Diego Zoo	Elad1			
EDA24	5485	103,780 (L)	Female	San Diego Zoo	Elad1			
EDA25	5492	682,196 (L)	Male	San Diego Zoo	Elad1			
EDA26	11,706	329	Male	San Diego Zoo	Elad1/Elad2	352	351	
EDA27	11,708	687,478 (L)	Female	San Diego Zoo	Elad2			

The Roca laboratory working ID, original facility ID, studbook number, and sex are listed for each deer as available. Only one haplotype is listed for homozygotes, both haplotypes are listed for heterozygotes. The sire and dam are listed for each individual as available. Individuals that are half-siblings (individuals that share only one biological parent) within the study samples are listed and sires and dams that are in the study samples are in boldface. The institutions are all accredited by the Association of Zoos and Aquariums (AZA). WCS is the Wildlife Conservation Society. Studbook numbers are from the Pere David's deer AZA regional studbook. Studbook numbers are not available for samples marked "(L)" for which local identification numbers are listed

Sequencher, which was also used to confirm open reading frames. Haplotype identity was verified using NCBI Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Confidence intervals for the haplotypes were calculated (Hazra 2017) using the following equation: $\hat{p} \pm z\sqrt{\frac{\hat{p}(1-\hat{p})}{n}}$. Sequences were translated using MEGA X v.10.1 (Kumar et al. 2018). The software PopART (with default parameters) was used to generate and illustrate a median-joining network (Bandelt et al. 1999; Leigh and Bryant 2015). The haplotype sequences generated by this study were deposited in GenBank (Accession Numbers MW804582 and MW804583).

Results

For all 27 Pere David's deer available, DNA was successfully extracted, and the complete open reading frame of *PRNP* was amplified and sequenced (Table 1). Four single nucleotide polymorphisms (SNPs) and two haplotypes were identified in the coding region. The two haplotypes detected were designated Elad1 and Elad2. The DNA sequence of haplotype Elad1 is the same as two previously reported Pere David's deer *PRNP* sequences (GenBank Accession Numbers: KC476497 and PRJNA256236). The Pere David's deer

PRNP haplotype Elad2 sequence is novel and has not been previously detected in any other cervid species, nor has its PrP amino acid sequence been previously reported.

Haplotype Elad1 was detected in 51 of 54 (0.944 ± 0.061 [95% confidence interval or CI]) phased Pere David's deer *PRNP* sequences, and thus had the highest frequency. Haplotype Elad2 had a much lower frequency and was represented by three of the 54 Pere David's deer *PRNP* sequences examined (95% CI 0.056 ± 0.0612). Samples of deer carrying Elad2 were extracted, amplified and sequenced a second time for verification. Haplotypes Elad1 and Elad2 differ at four nucleotide positions. One of the polymorphisms, c.183C>T is a synonymous SNP which has not been previously detected across cervids. The three other SNPs were non-synonymous SNPs encoding amino acid variation previously detected in other species of cervids (Table 2). In Elad2, SNP c.413A>G encodes serine (S) at codon 138, which is also present in many deer taxa (Table 2); whereas in Elad1, asparagine (N) is encoded at codon 138 (as reported in fallow deer and some caribou; Table 2). SNP c.624G>A encodes isoleucine (I) at codon 208 in Elad2,

whereas methionine (M) is encoded by Elad1. Both variants have been reported in red deer (*Cervus elaphus*), whereas methionine is common in other deer species (Table 2). SNP c.676C>G encodes glutamine (Q) at codon 226 in Elad2, whereas glutamic acid (E) is encoded by Elad1; both variants have been reported in red deer and sika deer (Table 2).

Amino acid sequences of PrP were aligned for some cervid taxa with *PRNP* haplotype sequence available in GenBank, along with Elad1 and Elad2 (Table 2). These sequences represent much of the reported PrP variation in cervids, and include PrP amino acid variants associated with reduced vulnerability to CWD in cervid taxa, as indicated in Table 2. Both of the Pere David's haplotypes encoded amino acids that had previously been associated with reduced vulnerability to CWD in other species.

For the cervid taxa shown in Table 2, a median-joining network (Fig. 1) was generated using *PRNP* sequences. The two Pere David's deer *PRNP* haplotypes were not most similar to each other. Instead, Elad1 was most similar to a *PRNP* sequence for fallow deer, while Elad2 was more similar to a *PRNP* sequence that is shared by Iberian red deer (*Cervus*

Table 2 Non-synonymous variation in select cervid taxa

Subfamily	Taxon	Common name	Designation	Codons													Source
				20	95	96	98	109	132	138	176	208	209	225	226		
Cervinae	<i>Elaphurus davidianus</i>	Pere David's deer	Elad1	D	Q	G	T	K	M	N	N	M	M	S	Q	This study	
	<i>Elaphurus davidianus</i>	Pere David's deer	Elad2	S	.	I*	.	.	E	This study	
	<i>Cervus canadensis</i>	Rocky Mountain elk (wapiti)		S	E	EU082291	
	<i>Cervus canadensis</i>	Rocky Mountain elk (wapiti)	L132	L	S	E	AF016228	
	<i>Cervus elaphus hispanicus</i>	Iberian red deer	226E	S	E	KT845864	
	<i>Cervus elaphus</i>	Red deer	TPQ	-	-	-	-	-	-	-	-	-	-	-	-	Robinson et al. (2019)	
	<i>Cervus elaphus</i>	Red deer	TPE	-	-	-	-	-	-	-	-	-	-	-	-	Robinson et al. (2019)	
	<i>Cervus elaphus</i>	Red deer		-	-	-	-	-	-	-	-	I	-	-	-	Kaluz et al. (1997)	
	<i>Muntiacus reevesi</i>	Reeves's muntjac		.	.	.	S	.	.	S	MK103020	
	<i>Cervus nippon</i>	Sika deer		S	E	AY679695	
	<i>Cervus nippon</i>	Sika deer	Haplotype 1	S	MK103018		
	<i>Dama dama</i>	Fallow deer		S	E	MK103017	
	Capreolinae	<i>Rangifer tarandus ssp.</i>	Caribou (Alaska)		S	-	Cheng et al. (2017)
		<i>Rangifer tarandus tarandus</i>	Reindeer (Europe)		S	D	MK097270
		<i>Odocoileus virginianus</i>	White-tailed deer	PrP variant A	S	MG856905
		<i>Odocoileus virginianus</i>	White-tailed deer	PrP variant C	.	.	.	S	.	.	S	MG856907
<i>Odocoileus virginianus</i>		White-tailed deer	PrP variant F	H	S	MG856910	
<i>Odocoileus virginianus</i>		White-tailed deer		S	Angers et al. (2010)	
<i>Odocoileus hemionus</i>		Mule deer		G	-	-	-	-	F	-	Jewell et al. (2005)	
<i>Odocoileus hemionus</i>		Mule deer		-	-	-	-	-	.	Angers et al. (2010)	
<i>Odocoileus hemionus hemionus</i>		Mule deer		S	AF009181	
<i>Capreolus capreolus</i>		European roe deer		S	MK103016	
<i>Alces alces alces</i>		Moose (European "elk")		Q	.	S	JQ290077	
<i>Alces alces shirasi</i>		Shiras moose		S	.	.	I	.	.	AY225485	
<i>Hydropotes inermis</i>		Chinese water deer	Haplotype 1	S	MK103024	

Amino acid variation in PrP (prion protein) is shown for select taxa within the family Cervidae notably those that have been investigated for alleles associated with chronic wasting disease (CWD) vulnerability. Codon positions are shown only if the amino acid varies among the listed deer. The reference is the translated haplotype Elad1, which is the most common haplotype in Pere David's deer (51 out of 54 sequences). Elad2 was the other haplotype found in Pere David's deer (3 out of 54 sequences). Amino acids that do not differ from the reference are denoted with a period; for those that differ the amino acid is listed. Dashes indicate missing information. Haplotype or protein variant designations from the original publications are listed. Haplotype Elad1 matches the *PRNP* haplotype of two Pere David's deer in GenBank (accession numbers KC476497 and PRJNA256236). For the listed Iberian red deer, Rocky Mountain elk and the sika deer with the Q226E substitution, the GenBank DNA haplotypes are identical. Amino acids previously associated with reduced vulnerability to CWD in a taxon are outlined. Amino acids that have been associated with reduced vulnerability of CWD in other taxa and are carried by the Pere David's deer are in boldface. Asterisk indicates that this amino acid found in Pere David's deer has been previously reported to be associated with the inability to propagate CWD in sheep and thus potentially prevents the propagation of CWD to cervids. The source column lists the GenBank accession number except for the cervid taxa for which a reference is listed, for which only some amino acids were reported. The Genbank accession numbers for Elad1 and Elad2 are MW804582 and MW804583, respectively

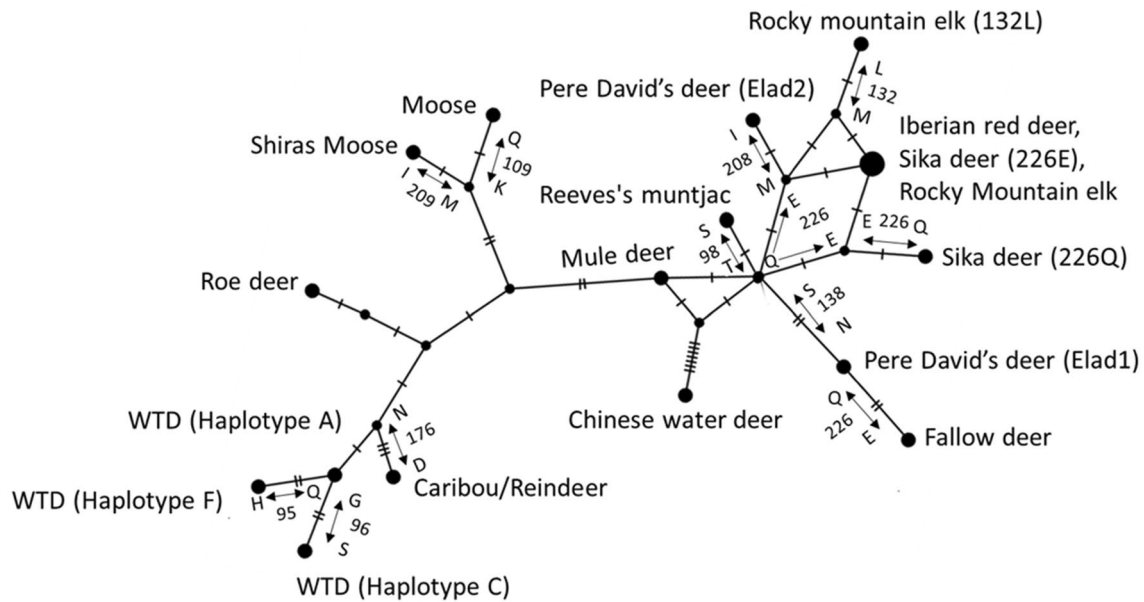


Fig. 1 Median-joining network of *PRNP* haplotypes for deer species in the subfamilies Capreolinae and Cervinae. Each labeled circle represents a distinct haplotype. Each hatch mark on the branches represents a nucleotide difference. For non-synonymous substitutions, the amino acids are listed along the branches with their codon number. Each of the Pere David's deer haplotypes (Elad1 and Elad2) is more similar to *PRNP* sequences in other species within the subfamily Cervinae than they are to each other. The fallow deer has been reported

elaphus hispanicus), Rocky Mountain elk (*Cervus canadensis*), and sika deer carrying the 226E substitution.

Discussion

No CWD infections have so far been reported among Pere David's deer. Although direct transmission from free-ranging deer may be possible at some facilities, the most likely potential means of transmission of CWD to Pere David's deer stocks in North America would be through feed or bedding. These can be contaminated by CWD-positive free-ranging North-American cervids, which can shed prions that persist in the environment and remain infectious (Mathiason et al. 2009; Henderson et al. 2015). There is also the concern that semen collected from infected deer can be an issue for captive facilities that use artificial insemination on their cervid populations, although the transmission risk is not currently known (Kramm et al. 2020).

Given the risk of transmission of CWD, it is worth comparing the amino acids sequence of Pere David's deer PrP with those of North American cervids undergoing outbreaks of CWD (Table 2, Fig. 1). In white-tailed deer, the SNP c.285A>C, which encodes a histidine (H) at codon 95, and the SNP c.286G>A, which encodes a serine (S) at codon 96,

to be less vulnerable to CWD because of the 138N codon that is fixed in the species (Rhyan et al. 2011; Haley et al. 2017), and is also present in Pere David's deer haplotype Elad1. WTD is the abbreviation for white-tailed deer. Haplotype designations are indicated in the parentheses beside several of the taxa. Identical *PRNP* haplotypes have been reported among Iberian red deer (KT845864), sika deer (AY679695), and Rocky Mountain elk (EU082291) (Table 2)

are associated with reduced vulnerability to CWD (Johnson et al. 2003; O'Rourke et al. 2004; Kelly et al. 2008; Brandt et al. 2015, 2018; Ishida et al. 2020). Mule deer with at least one sequence in which *PRNP* encodes phenylalanine (F) at codon 225 show a reduced frequency of CWD (Jewell et al. 2005; Geremia et al. 2015). Pere David's deer do not encode any of these three amino acids that are associated with reduced vulnerability to CWD in the native North American cervid species. However, this is not an indication of high vulnerability to CWD among Pere David's deer, as other codons present in the Pere David's deer sequence differed from those present in North American species.

The Elad1 and Elad2 haplotypes carried by Pere David's deer differ at four nucleotides, with one synonymous and three non-synonymous differences. The haplotype Elad1 was represented by 51 of the 54 *PRNP* sequences, and encoded asparagine (N) at codon 138, methionine (M) at codon 208 and glutamic acid (E) at codon 226; whereas haplotype Elad2, was represented by three of 54 *PRNP* sequences, encoded serine (S), isoleucine (I), and glutamine (Q), respectively (Table 2). All substitutions are conservative replacements (Betts and Russell 2003).

Both serine (S) and asparagine (N) at codon 138 are neutrally charged polar amino acids, but serine is smaller than asparagine, suggesting that this substitution could potentially

cause substantial configurational changes (Betts and Russell 2003). Asparagine at codon 138 (which is encoded by Elad1) has been associated with lower vulnerability to CWD in fallow deer (where it appears to be fixed) following experimental transmission (Rhyan et al. 2011; Robinson et al. 2019). CWD has been both experimentally transmitted (Mitchell et al. 2012) to and detected in free-ranging caribou/reindeer in Europe (*Rangifer tarandus tarandus*) (Benestad et al. 2016) but has only been experimentally transmitted to caribou in North America (*Rangifer tarandus* spp.) (Moore et al. 2016). The S138N substitution confers potentially reduced vulnerability in experimental inoculation studies of Norwegian reindeer (Mitchell et al. 2012) and North American caribou (Moore et al. 2016). Furthermore, in-vitro conversion of cervid PrP from elk, white-tailed deer, and mule deer (PrP variants from each of these taxa were cloned and expressed in cultured cells) into an infectious PrP^{CWD} form is reported to be more efficient for 138S than for 138N (Raymond et al. 2000). This suggests that the S138N substitution may reduce vulnerability to CWD in some deer species (Raymond et al. 2000). Additional studies would be needed to determine the actual role of 138N in CWD susceptibility in unexposed cervid taxa; several studies have examined the frequency of this potentially protective polymorphism (among others previously associated with a reduction in CWD vulnerability) in North American cervid populations (Happ et al. 2007; Cheng et al. 2017; Arifin et al. 2020). Elad1 encodes 138N, which suggests that it might also have a protective role in Pere David's deer. However, experiments using transgenic mice (LaFauci et al. 2006; Kurt et al. 2007) expressing Pere David's deer PrP, or a protein misfolding cyclic amplification experiment with Pere David's deer whole brain homogenate (Saborio et al. 2001), would have to be conducted to establish whether the substitution may provide actual protection among Pere David's deer.

Elad1 carries 208M as do the listed North American deer species, while Elad2 encodes isoleucine at codon 208 (Table 2). Both methionine (M) and isoleucine (I) are relatively unreactive and hydrophobic (Betts and Russell 2003). The substitution of methionine for isoleucine has been reported not to cause substantial structural changes to proteins (Ohmura et al. 2001). However, this substitution has been found to play a role in the interspecies prion transmission barrier between deer and sheep (Harrathi et al. 2019). While it is a possibility that 208I present in Elad2 may reduce vulnerability to CWD of Pere David's deer carrying this haplotype if exposed to CWD from North American deer that do not carry this substitution, the potential role of 208I in cervid-to-cervid transmission has not been examined. There is also the potential for reduced transmission between Pere David's deer carrying Elad1 and those carrying Elad2 due to potential barriers in transmission of CWD to PrP of variant configurations (Kurt and Sigurdson

2016). Both of these possibilities may suggest that the two haplotypes should be maintained in Pere David's deer populations, pending additional species-specific studies.

The final non-synonymous substitution within Pere David's deer is glutamine (Q) to glutamic acid (E) at codon 226. This is a common amino acid substitution, as both are polar and have similar physiochemical properties (Betts and Russell 2003). There is variation in the reported effects of the Q226E polymorphism in different cervid taxa. In red deer, deer homozygous for both the 226E and 226Q substitutions as well as heterozygous deer contracted CWD from an oral inoculation experiment with no significant difference in vulnerability (Balachandran et al. 2010). A study conducted in transgenic mice compared mice expressing a PrP variant found in Rocky Mountain elk with mice expressing a PrP variant found in both white-tailed deer and mule deer. It found that 226E carried by Rocky Mountain elk might allow for more efficient CWD prion propagation than the 226Q carried by mule deer and white-tailed deer (Angers et al. 2010). Yet, all reported fallow deer carry the 226E substitution (along with the 138N substitution), and are still resistant to direct infection by CWD from other cervids or indirect infection by a contaminated environment (Rhyan et al. 2011). As the role of the Q226E polymorphism on CWD vulnerability varies by species, studies for individual species are needed to determine what role if any it may play in vulnerability to CWD for each taxon. Pere David's deer Elad2 encodes 226E, which in native North American cervids has been reported only among Rocky Mountain elk; other North American taxa carry 226Q, which is also present in Elad1 (Table 2).

Despite the small number of founders, the two distinctive haplotypes have persisted into the modern population of Pere David's deer (Fig. 1). Both Elad1 and Elad2 share PrP amino acids with related cervid species (Table 2). The presence of trans-species polymorphisms, i.e., similar substitutions shared by different species, can be indicative of long-term balancing selection. The alleles that distinguish Elad1 from Elad2 were both likely present in an ancestor that gave rise to different species of the subfamily Cervinae (Klein et al. 1998; Charlesworth 2006; Koenig et al. 2019), and have since persisted in Pere David's deer. Balancing selection driven by transmissible spongiform encephalopathies has been identified for *PRNP* in other species, including human populations exposed to kuru (Mead et al. 2003), and sheep (*Ovis aries*) populations exposed to scrapie (Slate 2005). In North American elk, populations exposed to CWD may be under balancing selection at codon 132, which extends CWD latency but also potentially has negative fitness consequences (Monello et al. 2017). The presence of ancestral trans-species polymorphisms, and of multiple haplotypes in the small founder population, would both be consistent with the ancestors of Pere David's deer being subject to balancing

selection at the *PRNP* gene. If the persistence of Elad1 and Elad2 in Pere David's deer is due to balancing selection, this would suggest a fitness benefit for maintaining both haplotypes in the population. The frequency of Elad2 should be monitored so that it persists in Pere David's deer stocks. However, given the presence of 138N in Elad1 and the strong evidence for its protective role in fallow deer and caribou, it could be maintained as the more common haplotype.

The maintenance of both haplotypes in the Pere David's deer population is also recommended due to a likely reduction in potential intra-species transmission between deer carrying different PrP proteins, should CWD enter the population. Variation in CWD strain and PrP primary configuration have been shown to change PrP conversion rates and thus the presence of both PrP variants within a population could alter the effectiveness or speed of a CWD infection (Collinge and Clarke 2007; Kurt and Sigurdson 2016). Genetic management of captive populations of Pere David's deer to maintain both haplotypes should be considered an additional strategy for CWD management. This could be undertaken without affecting other goals, such as equalizing founder contributions; for example, if only one of two full siblings (which have identical ancestors) carries Elad2, then it could be favored over the other sibling for reproduction. Gametes from males that carry the Elad2 haplotype could also be collected and stored, to be used for the breeding of Pere David's deer in the future. The rarity of Elad2 suggests that increasing its frequency may have favorable fitness consequences should it become evident that either heterozygote advantage or rare allele effects are relevant to *PRNP*. Given the potentially protective role of 138N in Elad1, deer could also be selectively bred to maintain Elad2 in a heterozygous state.

Conclusion

We present an assessment of *PRNP* genetic polymorphisms in Pere David's deer, identifying two haplotypes including the novel Elad2. The large majority (94.4% of sequences) of Pere David's deer carried haplotype Elad1, which encodes asparagine (N) at codon 138 and glutamine (Q) at codon 226, both associated with a potential reduction in CWD vulnerability based on studies in other species. The remaining Pere David's deer *PRNP* sequences were Elad2, with isoleucine at codon 208, associated with lower vulnerability to CWD in another cervid species. Thus, it is possible that Pere David's deer may have reduced vulnerability to CWD. The trans-species polymorphisms detected across cervid species, and the presence of two divergent haplotypes among the small number of Pere David's deer founders are both potential signatures of ancestral balancing selection. Pere David's deer should be managed through breeding programs to prevent the loss

of rare haplotype Elad2, since the presence of two distinctive variants of PrP may help to limit the spread of CWD among the species, should there be an outbreak. Again, it must be emphasized that the studies establishing a role for these non-synonymous substitutions were conducted on other species, and a similar reduction in vulnerability to CWD would have to be established in Pere David's deer. Thus, all guidelines to reduce the potential spread of CWD in captive breeding programs for cervids should continue to be followed for Pere David's deer (Cullingham et al. 2020).

It seems unlikely, given the small number of founders, that additional *PRNP* haplotypes would be detected in Pere David's deer. However, additional individuals should be sequenced to identify those with Elad2 (or additional haplotypes). Wildlife ranches that have free-ranging herds are being considered as a source of breeding stock to add to the genetic diversity and conservation aims of zoos (Wildt et al. 2019), and could be assessed for genetic vulnerability to CWD. The inferences of the risk posed by CWD to Pere David's deer were based on studies conducted on other species. Additional in vivo or in vitro studies to directly examine the risk of various CWD strains to the species, would provide more direct information on the risk to Pere David's deer. Such studies could also help quantify the relative risk for homozygotes of Elad1 and Elad2, and for heterozygotes, which in turn could be used to determine ideal frequencies for the two haplotypes in the captive breeding stock of Pere David's deer. Finally, similar surveys of *PRNP* genetic variation could guide the management of other endangered cervid taxa.

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Data availability *PRNP* haplotype sequences for Pere David's deer have been deposited in GenBank under accession numbers MW804582 and MW804583. Sample information is listed in Table 1.

Code availability Not applicable.

Declarations

Conflict of interests The authors declare no conflict of interest in presenting this publication.

Ethics approval Research was conducted under the Illinois Institutional Animal Care and Use Committee protocol 18212. Individual material transfer agreements are in place for each institution that contributed samples, and all terms were adhered to.

Consent to participate (include appropriate statements) Not applicable.

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