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Excessive replacement changes drive evolution of global sheep prion protein (*PRNP*) sequences

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Sheep prion protein (*PRNP*) is the major host genetic factor responsible for susceptibility to scrapie. We aimed to understand the evolutionary history of sheep *PRNP*, and primarily focused on breeds from Turkey and Ethiopia, representing genome-wise ancient sheep populations. Population molecular genetic analyses are extended to European, South Asian, and East Asian populations, and for the first time to scrapie associated haplotypes. 1178 *PRNP* coding region nucleotide sequences were analyzed. High levels of nucleotide diversity driven by extensive low-frequency replacement changes are observed in all populations. Interspecific analyses were conducted using mouflon and domestic goat as outgroup species. Despite an abundance of silent and replacement changes, lack of silent or replacement fixations was observed. All scrapie-associated haplotype analyses from all populations also showed extensive low-frequency replacement changes. Neutrality tests did not indicate positive (directional), balancing or strong negative selection or population contraction for any of the haplotypes in any population. A simple negative selection history driven by prion disease susceptibility is not supported by the population and haplotype based analyses. Molecular function, biological process enrichment, and protein-protein interaction analyses suggested functioning of PRNP protein in multiple pathways, and possible other functional constraint selections. In conclusion, a complex selection history favoring excessive replacement changes together with weak purifying selection possibly driven by frequency-dependent selection is driving *PRNP* sequence evolution. Our results is not unique only to the Turkish and Ethiopian samples, but can be generalized to global sheep populations.

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INTRODUCTION

The prion protein gene (*PRNP*) is the major host genetic factor responsible for susceptibility to transmissible spongiform encephalopathies (TSEs) (Prusiner 1998). TSEs are heterogeneous lethal neurodegenerative diseases observed in a variety of mammals that are of great public health and economic concern. For many hundreds of years, sheep (*Ovis aries*) has been the most commonly affected small ruminant by prion diseases such as scrapie (Hunter 1997b; Hunter 1997a). Polymorphisms in the sheep *PRNP* gene, primarily the haplotypes formed by codons 136 (A and V), 154 (R and H), and 171 (Q, R, and H), have been associated with increased or decreased susceptibility to natural scrapie (Bossers et al. 2000; Goldmann et al. 1990). Consequently, there is interest in identifying the evolutionary forces that shape the molecular evolution of *PRNP*.

Both the origins and continuing presence of high-frequency scrapie susceptible genotypes in modern sheep have been investigated. Some genetic studies of *PRNP* focused mostly on sequence variation and the effect of genetic variation on susceptibility/resistance to TSEs within a species (Heaton et al. 2003; Hills et al. 2003; Frootan et al. 2012; Teferedegn et al. 2020a; Yaman et al. 2015; Uchida et al. 2014; Stepanek and Horin 2017; Meydan et al. 2012), whereas others examined the molecular evolution of *PRNP* via between species comparisons (Rongyan et al. 2008; Slate 2005; van Rheede et al. 2003; Wopfner et al. 1999). Multiple, sometimes contrasting, selection scenarios such as purifying, balancing, and positive selection on *PRNP* have been proposed (Rongyan et al. 2008; Slate 2005; Woolhouse et al. 2001). Lack of appropriate geographic sampling can be an important, but overlooked, reason for the discordant selection explanations. So far, within species *PRNP* genetic analyses have been conducted with rather limited sequence samples mostly derived from sequence databases without much depth of geographic sampling. Therefore, a comprehensive molecular population genetic analyses of *PRNP* sequences sampled from diverse geographic regions is necessary to understand the evolutionary history of *PRNP* gene.

Whether scrapie-like diseases have been a major selective force on the whole evolutionary history of *Ovis aries* or scrapie-based selection has become a major selective force much after the domestication should influence the pattern of sequence variation in *PRNP* differently. A comprehensive understanding of the evolutionary history of *PRNP* in domesticated sheep can only be possible by sampling contemporary sheep populations from geographic regions where domestication first happened. Whether suggested historical purifying (negative) selection or balancing selection has been shaping the genetic diversity at *PRNP* can be more thoroughly tested by investigating the genome-wise ancient

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populations, as natural selection had more time (generations) to act on the scrapie susceptible and resistant alleles.

Sheep is one of the first domesticated livestock in Southwest Asia, mainly the Fertile Crescent region (Fuks and Marom 2021; Zeder 2008; Bruford et al. 2003). Therefore Turkey, where the southeast region is part of the Fertile Crescent, is one of the most likely geographic regions where contemporary domesticated livestock still have evolutionary history signals of natural selection (or lack of it) going back to pre-domesticated and early domestication periods. Ethiopia is believed to be one of the major gateways for domestic sheep migration from Southwest Asia to Africa, where the earliest African domestic sheep remains have been dated going back to thousands of years ago, and has diverse indigenous sheep populations (Muigai and Hanotte 2013; Abdurehman 2019).

In this study we aimed to decipher the selective forces that have been shaping the evolutionary history of sheep *PRNP* by conducting comprehensive molecular population genetic analyses of *PRNP* sequences from different breeds (localities), and also scrapie resistance/susceptibility associated haplotypes from Ethiopia, Turkey, Europe, South Asia, and East Asia, where sheep domestication and breeding histories are rather different. More specifically, we wanted to see whether strong purifying selection against the scrapie susceptible haplotype(s), or positive selection favoring the resistant haplotype(s) have been shaping the molecular evolution of PRNP. The study is designed to capture the *PRNP* sequence diversity primarily from Turkey (one of the most likely centers for the origin of contemporary domestic sheep) and Ethiopia (one of the major gateways for domestic sheep migration from Southwest Asia to Africa), do comprehensive population genetic analyses with these sequences, and then extend the analyses to high quality sequences that have already been collected from Europe, South East Asia and East Asia in order to test the validity and generality of results obtained from Turkish and Ethiopian samples.

Our findings indicate that scrapie has not been a major selective force shaping the molecular evolutionary history of sheep PRNP. A complex selection history favoring excessive replacement changes together with weak purifying selection possibly driven by frequency dependent selection is driving the *PRNP* sequence evolution. Also, our conclusions have implications for the possible counterproductive consequences of popularized breeding policies aiming to eradicate scrapie susceptible alleles.

MATERIALS AND METHODS

Generation of DNA sequence data

The origin of Ethiopian and Turkish samples, and molecular methods used for data generation was previously reported (Teferedegn et al. 2020a; Ün et al. 2008). Briefly, genomic DNA was isolated from blood samples, and the coding region of *PRNP* was PCR amplified and sequenced using forward 5′-TCTGCAAGAAGCGACCAAAAC-3′ and reverse 5′-CACAGGAGGGGAAGAAAAGAGG-3′ primers for the Ethiopian, and forward 5′-AAAGCCACATAGGCAGTTG-3′ and reverse 5′-AATGAGGAAAGAGATGAGGAG-3′ primers for the Turkish samples. Forward 5′-AAAGCCACATAGGCAGTT-3′, and reverse 5′-AATGAGGAAAGAGATGAGGAG-3′ primers were used for the domestic goat (*Capra hircus*) samples. Ethiopian, Turkish, and goat sample sequences are deposited to NCBI with the following accession codes. Ethiopian MN834021–MN834117; Turkish JX187517.1–JX187538.1; goat (*Capra hircus*) MN795374–MN795447.

Whereas DNA sequence data for Ethiopian, Turkish, and goat samples were generated experimentally, other world-wide *PRNP* DNA sequences that were used for population genetics comparisons are retrieved from NCBI database with the following accession codes. European AF180389.1–AF195247.1, AJ000679.1–AJ67988.1, MH934799.1–MH4909.1; Indian DQ013286.1–DQ885794.1; Chinese AB307632.1–AB373810.1, HM639748.1-HM803994.1, HQ197668.1–HQ897614.1, JF310745.1–JF514143.1; Mouflon (*Ovis aries musimon*) FJ792605, FJ792606 (Supplementary Table 1).

PHASE algorithm (Stephens and Donnelly 2003; Stephens et al. 2001) was used to construct haplotypes from aligned sequences. Scrapie resistant and susceptible haplotypes are classified according to the literature (Goldmann et al. 1990; Woolhouse et al. 2001; Hunter 1997b). ARR is the resistant haplotype; VRQ is the susceptible haplotype; ARQ, ARH, and AHQ are associated with little to moderate resistance.

Population genetics, statistics and bioinformatics analyses

Population genetic summary statistics for nucleotide diversity included segregating sites (S), total number of mutations (Eta), number of haplotypes (Nei 1987), haplotype diversity (Nei 1987), nucleotide diversity (Pi, π) (Nei 1987), average number of nucleotide differences Watterson theta (θ) (Nei 1987; Watterson 1975). Recombination per gene and per adjacent sites were also estimated (Hudson and Kaplan 1985; Hudson 1987). Allele frequency spectrum based neutrality tests included Tajima's D (Tajima 1989), Fu and Li's D* (Fu and Li 1993), Fu and Li's F* (Fu and Li 1993), Fu's Fs (Fu 1997), and Ramos-Onsins and Rozas R2 (Ramos-Onsins and Rozas 2002). McDonald and Kreitman's Test (McDonald and Kreitman 1991), Neutrality index (NI) (Rand and Kann 1996), Alpha (α) value (Fay et al. 2001), and Direction of Selection (DoS) (Stoletzki and Eyre-Walker 2011) was used for intra- and interspecific population sequence comparisons. All population genetic parameter estimations and tests were implemented by DnaSP 6 (Rozas et al. 2017). Haplotype networks are constructed using Popart 1.7 (Population Analyses with Reticulate Trees) software package (Leigh and Bryant 2015) using Nexus files as input data. Phylogenetic analyses were conducted in MEGA X (Kumar et al. 2018). Distribution of scrapie risk-associated haplotypes among different breeds are compared with Chi-square tests. For all statistical analysis, a P value of less than 0.05 was taken as a statistically significant result. Statistical tests and graphing is conducted with R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria, https://www.r-project.org).

Molecular function and biological pathway analyses

Molecular function enrichment, biological process enrichment, and protein-protein interaction analyses were conducted using PANTHER (http://pantherdb.org/) and STRING (Szklarczyk et al. 2019) (https://string-db.org) online databases.

Ethical approval

Animals were treated with great care and sample was taken according to the institute guidelines. For Turkish samples, all animal procedures in the study were reviewed and approved by the local ethics committee of Sheep Breeding and Research Institute (Approval Number: 1282412). Ethical approval is deemed unnecessary according to the Ethiopian National Research Ethics Review Guideline/EFDRE ministry of science and Technology Sep 2014 5th ed. Article 8.3.5.1, 10.2 and 10.5.1. Consent was granted to take blood samples from the respective regional state livestock development and promotion offices NS/AR/U-01/42/2010 and NS/AR/U-01/41/2010. Genetic material export permit was assured from Ethiopian Biodiversity Institute, Ref. No. EBI71/943/2018.

RESULTS

Intraspecific analyses

Molecular population genetic analyses aiming to examine the evolutionary history of *PRNP* started with population-based analyses primarily focusing on the sequenced Turkish and Ethiopian samples. To see whether the population genetic parameter estimates are unique only to the sampled Turkish and Ethiopian populations, or represent a more general global pattern, analyses are extended to sequences from Europe, South Asia, and East Asia that were downloaded from the NCBI database.

In total 1178 sequences with a median of 507 nucleotides of the PRNP third exon from Turkey, Ethiopia, Europe, South Asia, and East Asia regions were analyzed. In all populations the number of replacement (nonsynonymous) polymorphisms were higher than synonymous polymorphisms. Considering all sites, Turkish samples had the highest nucleotide diversity indicated by the theta (θ) parameter estimate, which is based on the number of segregating sites.

However, the other nucleotide diversity parameter estimate pi (π), which is based on average pairwise nucleotide differences, indicated that Ethiopian samples had the highest nucleotide

diversity. Haplotype diversity was also highest in Ethiopian followed by Turkish samples. Turkish samples had the highest number of replacement polymorphisms followed by Chinese samples (Table 1). When individual populations within Ethiopia (Afar, Washera, and Menz samples) were compared, no one population showed marked difference compared to others based on number of replacement and silent (synonymous) polymorphisms, nucleotide or haplotype diversity (Table 1), suggesting similar evolutionary population history and structure. Individual Turkish populations, on the other hand, showed rather different numbers of replacement and synonymous polymorphisms, nucleotide and haplotype diversity estimates (Table 1), suggesting different molecular evolutionary patterns probably shaped by different breeding histories.

Next, a number of population genetic tests, primarily based on the allele frequency spectrum distributions, were conducted to examine whether the observed nucleotide diversity patterns in each population follows neutral expectations (i.e., no selection and constant population size) or shows statistically significant deviations from neutral expectations indicating selection or drastic demographic changes. When all samples (from all populations) were considered as one big panmictic population and analyzed together, statistically significant negative Tajima's D (TD) results indicated excess of low frequency (rare) polymorphisms in the sequence data (Table 2). Conducting the TD test for synonymous and nonsynonymous sites separately gave negative TD results for both site classes, however, only the test result for the nonsynonymous sites were statistically significant indicating excess of low-frequency replacement changes in the sequence data (Table 2). Excess of low-frequency replacement polymorphisms were suggested by negative test results in all populations, and breeds, though most of the test results were not statistically significant (Table 2). Excess of low-frequency polymorphisms suggested by TD and Fu-Li tests can be due to rapid population growth, background selection, or purifying selection acting on *PRNP*. However, Ramos-Onsins and Rozas R2 and Fu's Fs test results, which are the preferred tests for detecting population size changes, did not indicate drastic demographic changes in any population or breed. For background selection to have generated the observed excess of singleton changes would require a complete lack of recombination in the PRNP gene, and around its genome region. However, the minimum number of recombination events and recombination between adjacent sites estimates are greater than zero in all populations (Supplemental Table 2). These recombination parameter estimates in independent populations, which should be an underestimate of actual recombination in natural populations, suggest that there is ample recombination going on in the *PRNP* gene. Therefore it is very unlikely that a strong background selection is operating on *PRNP*. On the other hand, the negative TD and Fu-Li test results are primarily driven by nonsynonymous (replacement) polymorphisms. So, a weak purifying selection acting on the segregating slightly deleterious *PRNP* replacement changes could be driving the observed allele frequency spectrum in all populations, and breeds.

Interspecific analyses

To further test possible selection scenarios on *PRNP*, a McDonald-Kreitman test approach was adopted comparing the ratio of nonsynonymous to synonymous variation within sheep samples to the ratio of nonsynonymous to synonymous variation between sheep and mouflon (*Ovis aries musimon*), and sheep and goat (*Capra hircus*) sequences. When all sheep sequences were pooled no silent or replacement nucleotide change fixations were observed between the sheep and mouflon samples (Table 3). The same lack of fixed differences was evident when each population sample was analyzed separately. When similar analyses were conducted against the goat *PRNP*, still lack of silent or replacement fixations was rather evident despite the abundance

Table 1. Population genetic summary statistics for *PRNP* nucleotide diversity among examined world sheep populations.

Population	Sample size	Total bases	Syn. sites	Nonsyn. sites	S (Eta)	Syn. Pol.	Rep. Pol.	θ (JC) All sites	π (JC) All sites	π (JC) Syn. sites	π (JC) Nonsyn. sites	H	H_d
All	1178	486	110.2	375.8	43 (47)	14	31	126	32	37	31	74	0.85
Ethiopian	194	507	115.7	391.3	14 (16)	6	10	54	45	11	28	35	0.91
Menz	78	507	115.9	391.1	13 (15)	6	9	60	41	10	25	21	0.87
Washera	70	507	115.4	391.6	11 (13)	5	8	53	42	9	30	23	0.91
Afar	46	507	115.6	391.4	9 (10)	3	7	45	45	11	26	12	0.86
Turkish	263	618	144.1	470.9	35 (36)	10	26	95	27	26	28	37	0.83
İvesi	93	598	139.9	433.1	15 (15)	4	11	49	29	16	35	22	0.86
Kıvırcık	87	618	147.5	467.5	10 (10)	3	7	32	17	6	20	11	0.73
Çine Çapan	25	618	148.3	469.7	5 (5)	1	4	21	18	22	17	6	0.78
Karakaçan	24	618	148.4	469.6	5 (5)	0	5	22	15	0	19	6	0.70
İmroz	18	618	148.4	469.7	7 (7)	1	6	33	21	8	25	6	0.70
Karacabey merino	10	618	147.8	467.2	10 (10)	2	8	57	54	44	57	5	0.82
Sakız	6	618	147.0	468.0	8 (8)	2	6	57	47	46	47	4	0.80
European	235	507	115.6	391.4	10 (11)	1	9	36	26	1	33	12	0.77
Indian	236	507	115.5	391.5	14 (14)	2	12	46	35	64	27	23	0.83
Chinese	250	507	115.4	391.6	23 (24)	10	14	78	29	41	26	29	0.79

Sample size show the number sequences analyzed.
θ and π values represent percent sequence diversity and for exact estimates table values should be multiplied by 10^{-4}. Turkish İvesi and Sakız breeds are also known as Awassi and Chios, respectively.
Syn Synonymous sites; *Nonsyn* Nonsynonymous sites; *S* Number of segregating sites; *Eta* Number of mutations; *Syn. Pol.* Number of synonymous polymorphisms; *Rep. Pol.* Number of replacement (nonsynonymous) polymorphisms; *JC* Jukes-Cantor correction applied estimates; *H* Number of haplotypes; *Hd* Haplotype diversity.

Table 2. Neutrality tests summary statistics for PRNP among examined world sheep populations.

Population	TD	TD – Syn.	TD – Nonsyn.	Fu-Li's D*	Fu-Li's F*	Fu-Li's D	Fu-Li's F	Fu's Fs	R2
All	−2.14**	−1.57	−2.10**	0.06	−1.09	0.06	−1.10	−101.3	0.02
Ethiopian	−0.46	0.71	−1.17	1.61*	0.99	1.64*	1.01	−26.12	0.08
Menz	−0.91	0.48	−1.15	0.04	−0.35	0.02	−0.38	−12.73	0.08
Washera	−0.65	0.25	−1.15	0.41	0.07	0.41	0.06	−16.33	0.10
Afar	−0.018	1.70	−0.96	0.80	0.63	0.82	0.64	−3.28	0.12
Turkish	−1.85*	−1.76*	−1.62	−2.60*	−2.82*	−2.69*	−2.88**	−35.04	0.02
İvesi	−1.15	−1.42	−0.80	−0.55	−0.91	−0.59	−0.96	−15.65	0.06
Kıvırcık	−1.26	−1.56	−0.80	−2.05	−2.11	−2.15	−2.19	−5.27	0.05
Çine Çaparı	−0.41	0.43	−0.66	−0.52	−0.57	0.29	0.10	−1.53	0.11
Karakaçan	−0.93	–	−0.93	−1.34	−1.41	−0.59	−0.82	−2.26	0.09
İmroz	−1.25	−1.16	−1.07	−1.12	−1.33	−1.34	−1.57	−1.69	0.10
Karacabey merino	−0.31	−0.69	−0.14	−0.03	−0.11	−0.21	−0.28	0.54	0.15
Sakız	−1.07	−1.13	−0.93	−1.06	−1.14	−1.73	−1.89	0.15	0.19
European	−0.74	–	−0.74	1.37	0.74	1.39	0.87	−3.06	0.07
Indian	−0.56	1.49	−1.09	0.21	−0.10	0.21	−0.11	−11.67	0.06
Chinese	−1.93*	−1.49	−1.79*	1.39	0.17	1.42	0.17	−23.65	0.03

TD Tajima's D test; *Syn* Synonymous sites; *Nonsyn* Nonsynonymous sites; *R2* Ramos-Onsins and Rozas R2 estimate. Turkish İvesi and Sakız breeds are also known as Awassi and Chios, respectively.
*$P < 0.05$; **$P < 0.01$.

Table 3. Intra- and interspecific PRNP sequence comparisons using mouflon (*Ovis aries musimon*) and goat (*Capra hircus*).

	Mouflon						Goat					
	Silent	Replacement	P	NI[a]	Alfa[b]	DoS[c]	Silent	Replacement	P	NI[a]	Alfa[b]	DoS[c]
All samples												
Polymorphic	12	27	–[d]	–[d]	–[d]	−0.69	12	30	–[d]	–[d]	–[d]	−0.71
Fixed	0	0					0	0				
Ethiopian												
Polymorphic	5	8	–[d]	–[d]	–[d]	−0.38	5	11	–[d]	–[d]	–[d]	−0.69
Fixed	0	0					0	0				
Turkish												
Polymorphic	7	25	–[d]	–[d]	–[d]	−0.78	7	28	0.99	1	0	0
Fixed	0	0					1	4				
European												
Polymorphic	2	7	–[d]	–[d]	–[a]	−0.78	0	12	0.08	–[d]	–[d]	−1
Fixed	0	0					1	0				
Indian												
Polymorphic	2	12	–[d]	–[d]	–[d]	−0.86	2	17	0.15	–[d]	–[d]	−0.89
Fixed	0	0					1	0				
Chinese			–[d]	–[d]	–[d]	−0.57						
Polymorphic	9	12					9	17	0.37	–[d]	–[d]	−0.65
Fixed	0	0					1	0				

[a]NI: Neutrality Index (departure from neutrality).
[b]Alpha (α): proportion of substitutions driven by positive selection.
[c]DoS: Direction of selection (difference between the proportion of substitutions and polymorphisms that are replacements).
[d]Statistical test cannot be conducted due to zero count cells.

of silent and replacement changes (Table 3). For Turkish samples the neutrality index (NI) was one, and alpha value that estimates adaptive substitutions was zero. For all other populations NI and alpha could not be estimated due to lack of replacement fixations. Direction of selection (DoS) tests were negative but close to zero estimates in all populations clearly suggesting lack of positive (directional) or strong negative (purifying) selection on *PRNP* (Table 3). However, the abundance, but lack of fixations, of replacement polymorphisms and slightly negative DoS tests suggest weak purifying selection acting on the *PRNP* sequences.

Scrapie related haplotype analyses

If scrapie is a major driver of *PRNP* sequence evolution than scrapie-associated haplotypes should be the main target of

selection. Therefore, following population-based intraspecific and species-based interspecific analyses, population genetic analyses are extended to scrapie associated haplotypes in Turkish and Ethiopian samples. All scrapie-associated haplotypes were observed in the Turkish and Ethiopian samples, except the scrapie susceptible VRQ haplotype which was absent in the Ethiopian samples. Whereas ARQ was the most common haplotype in both populations, the scrapie resistant ARR was the second most common haplotype in Turkish populations, and it was the least common haplotype in Ethiopian samples (Table 4). Although the distribution of haplotypes among Ethiopian, and Turkish sheep breeds was not of equal proportions (Chi-square P values < 0.05, Supplementary Fig. 1), most probably driven by small sample size in certain breeds (i.e., Karacabey merino and Sakız), no haplotype was unique to any breed. This observation clearly suggests that haplotype sequences analyzed in this study have been interacting with and evolving in diverse genomic backgrounds, and haplotype analysis results are not specific to any breed, and not just shaped by the evolutionary and breeding history of a specific breed.

Any strong selection on scrapie-associated haplotypes should influence the topology of their phylogenetic tree and haplotype network. However, dendograms of Neighbor-joining phylogenetic trees of Ethiopian and Turkish samples showed that haplotypes are not separated from each other via distinct nodes. None of the branches or nodes are dominated by or specific to any haplotypes (or any breeds), rather they indicate sharing of alleles (Supplementary Fig. 2). There are no distinct phylogroups or clades unique to any haplotype. Moreover, tip branch lengths are very short indicating excess of rare (or singleton) variants in the Ethiopian and Turkish samples (Supplementary Fig. 2). Haplotype network analyses of Ethiopian and Turkish samples support the observations from Neighbor-joining dendogram analyses. None of the haplotypes are represented in only a single node, rather they are distributed throughout the highly interconnected network of nodes (Supplementary Fig. 3). The highly interconnected topology of the haplotype networks also support ongoing recombination between the sequences as had been suggested by recombination parameter estimates (Supplemental Table 2). Taken together, phylogenetic and haplotype network analyses do not support a strong positive or negative selection, or a selective sweep in any haplotype in the Ethiopian and Turkish samples.

Molecular population genetic summary statistics and neutrality tests are also conducted for scrapie-associated haplotypes. Among Ethiopian samples ARQ, ARH, and AHQ haplotypes showed high haplotype and nucleotide diversities primarily driven by rare or singleton changes suggested by negative TD and FL (Fu-Li) tests (Table 4). Among Turkish samples whereas the resistant ARR haplotype showed the highest nucleotide diversity based on the number of segregating sites (θ parameter), the AHQ haplotype showed the highest diversity based on average pairwise nucleotide difference (π parameter) estimate, and also the highest haplotype diversity (Table 4). For all haplotypes the nucleotide diversities were primarily driven by rare or singleton changes suggested by negative TD and FL tests (Table 4). Population genetic tests did not suggest positive or negative selection for any of the haplotypes in Ethiopian and Turkish samples. Fu's Fs, and Ramos-Onsins and Rozas R2 also did not indicate significant population expansion or contraction for any haplotypes in these populations.

To test whether these observations are unique only to Ethiopian and Turkish samples or can reflect a more general evolutionary trend in other populations, the analyses are extended to other world population samples, where the evolutionary histories can be different. Overall, nucleotide and haplotype diversity, and other parameter estimates in European, Indian, and Chinese samples were similar to that of Ethiopian and Turkish samples (Table 4). Extending the analyses to other populations highlights genetic diversity in other haplotypes in addition to the ARQ, ARH, and AHQ. For example, the susceptible VRQ haplotype has considerable genetic diversity in both the Turkish, and European samples driven by low frequency/rare replacement polymorphisms (Table 4). Interestingly, the replacement (nonsynonymous) site diversity of the susceptible VRQ is higher than the scrapie resistant ARR haplotype not only the in Turkish and Chinese samples but also in the European samples, where scrapie susceptible haplotype eradication programs have been implemented. This observation does not suggest a strong purifying or negative selection against VRQ, and does not support an evolutionary history favoring the ARR. Moreover, Fu's Fs, and Ramos-Onsins and Rozas R2 did not indicate significant population expansion or contraction for any haplotypes in any of the populations (Table 4). Comparing the ratio of nonsynonymous site pairwise nucleotide diversity (π_{NS}) to nonsynonymous site plus synonymous site pairwise nucleotide diversity [$\pi_{NS}/(\pi_{NS} + \pi_S)$] shows that majority of the variation among haplotypes are driven by replacement changes in most populations (Fig. 1). This observation suggests that presence of extensive replacement changes among all haplotype backgrounds is not just specific to Ethiopian or Turkish samples, but common to all populations examined from three continents.

Most probably these results are an underestimate of the *PRNP* genetic diversity present in world-wide populations. Some haplotypes, such as VRQ, was absent or represented by very few sequences in some populations. Despite this, abundant genetic diversity was observed in all scrapie-associated haplotypes. Increasing the number of sequences from diverse populations can increase genetic diversity estimates, primarily the nonsynonymous site diversity, and further support our suggestion of an evolutionary history favoring extensive replacement changes in *PRNP*.

DISCUSSION

We aimed to uncover the evolutionary factors shaping the molecular evolution of *PRNP* by conducting comprehensive molecular population genetic analyses with *PRNP* nucleotide sequences of sheep breeds from Ethiopia and Turkey, and other world populations. Ample nucleotide and haplotype diversity primarily driven by replacement polymorphisms was observed in all sheep populations, being slightly higher for the Turkish and Ethiopian samples. Population genetic analyses did not indicate clear negative, purifying or balancing selection signals in any of the populations or scrapie associated *PRNP* haplotypes. Even in European samples, for which one might expect signs of negative selection and reduction in nucleotide diversity due to decades-long artificial selection against scrapie susceptible genotypes, all diversity estimates were comparable to other populations, and no negative or purifying selection, drastic population contraction was evident.

Similar to population-based analyses, scrapie-associated haplotype analyses did not indicate strong negative, purifying or balancing selection in any of the haplotypes. There is no evidence for strong purifying selection against the scrapie susceptible haplotype(s), or positive selection favoring the resistant haplotype(s).

Population-level analyses were followed by species-level comparisons. Intra- and interspecific *PRNP* sequence comparisons using mouflon as an outgroup still showed abundance of synonymous and replacement changes arguing against negative selection, but also did not suggest adaptive evolution of *PRNP*, at least in the sheep clade, due to lack of fixations. One can argue that lack of fixation in any group with respect to mouflon can be an indication of recent species separation and ongoing interbreeding. However, very similar results were also obtained using goat as an outgroup species. Neutrality index, alpha value, and direction of selection (DoS) estimates also suggested lack of strong negative, purifying or positive selection. Taken together, very small number of fixations but abundance of polymorphisms

Table 4. Population genetic summary statistics for *PRNP* sequences stratified by scrapie susceptibility haploytpes.

Population	Sample size	S (Eta)	Syn. Pol.	Rep. Pol.	θ (JC) All sites	π (JC) All sites	π (JC) Syn. Sites	π (JC) Nonsyn. Sites	H	H_d	TD	FL-D*	FL-F*	Fu F_s	R2
Ethiopian															
All	194	14 (16)	6	10	54	45	11	28	35	0.91	−0.46	1.61*	0.99	−26.1	0.08
ARR	3	0 (0)	0	0	0	0	0	0	1	0.00	–	–	–	–	–
ARQ	128	12(14)	6	8	51	38	11	17	18	0.83	−0.70	0.95	0.41	−7.4	0.08
ARH	53	9 (10)	4	6	44	25	89	7	13	0.79	−2.01*	−2.33*	−2.31*	−7.8	0.07
AHQ	10	6 (7)	3	4	49	42	90	28	6	0.89	−0.58	−0.13	−0.27	−1.7	0.17
VRQ	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Turkish															
All	263	35 (36)	10	26	95	28	27	28	37	0.83	−1.85*	−2.60*	−2.82*	−35.0	0.02
ARR	70	15 (16)	3	12	54	11	7	12	9	0.27	−2.31**	−3.65**	−3.79**	−5.57	0.04
ARQ	150	13 (13)	0	13	39	13	0	16	17	0.52	−1.57	−1.06	−1.56	−15.9	0.03
ARH	26	12 (12)	3	9	51	27	33	25	11	0.75	−1.62	−1.67	−1.94	−6.02	0.06
AHQ	6	5 (5)	1	4	36	34	40	32	4	0.80	−0.31	−0.21	−0.25	−0.44	0.19
VRQ	11	6 (6)	1	5	33	20	13	23	4	0.49	−1.57	−1.62	−1.81	−0.29	0.13
European															
All	235	10 (11)	1	9	33	26	1.2	33	12	0.77	−0.74	1.37	0.74	−3.06	0.07
ARR	98	3 (3)	0	3	11.5	1.6	0	2.1	3	0.04	−1.55	−1.99	−2.17	−2.72	0.05
ARQ	88	8 (8)	0	8	31	22	0	28	9	0.77	−0.75	0.48	0.07	−2.72	0.07
ARH	6	1 (1)	1	0	9	11	46	0	2	0.53	0.85	1.05	1.03	0.63	0.27
AHQ	6	0 (0)	0	0	0	0	0	0	1	0.00	–	–	–	–	–
VRQ	37	3 (3)	0	3	14	7	0	9	4	0.21	−1.12	−0.33	−0.65	−1.82	0.07
Indian															
All	236	14 (14)	2	12	46	35	64	27	23	0.83	−0.56	0.21	−0.10	−11.7	0.06
ARR	31	2 (2)	0	2	10	4	0	5	3	0.19	−1.26	−0.75	−1.03	−1.7	0.09
ARQ	115	6 (6)	2	4	22	15	33	9	9	0.49	−0.77	1.11	0.57	−4.2	0.06
ARH	63	9 (9)	2	7	38	31	87	15	11	0.83	−0.48	−0.04	−0.22	−3.5	0.09
AHQ	23	4 (4)	2	2	21	38	89	24	5	0.82	2.16*	1.09	1.61	0.81	0.24
VRQ	2	0 (0)	0	0	0	0	0	0	1	0.00	–	–	–	–	–
Chinese															
All	250	23 (24)	10	14	78	29	41	26	29	0.79	−1.93*	1.39	0.17	−23.7	0.03
ARR	55	12 (12)	5	7	52	20	44	12	10	0.52	−1.82*	−0.18	−0.87	−5.3	0.04
ARQ	148	18 (18)	7	11	64	24	54	15	20	0.58	−1.72	1.69*	0.46	−15.1	0.03
ARH	29	3 (3)	0	3	15	7	0	9	3	0.20	−1.33	−0.25	−0.65	−0.72	0.08
AHQ	15	0 (0)	0	0	0	0	0	0	1	0.00	–	–	–	–	–
VRQ	3	1 (1)	0	1	13	13	0	17	2	0.67	–	–	–	–	0.47

Sample size show the number sequences analyzed.

θ and π values represent percent sequence diversity and for exact estimates table values should be multiplied by 10^{-4}. ARR is the resistant haplotype; VRQ is the susceptible haplotype; ARQ, ARH, and AHQ are associated with little to moderate resistance.

Syn Synonymous sites; *Nonsyn* Nonsynonymous sites; *S* Number of segregating sites; *Eta* Number of mutations; *Syn. Pol.* Number of synonymous polymorphisms; *Rep. Pol.* Number of replacement (nonsynonymous) polymorphisms; *JC* Jukes-Cantor correction applied estimates; *H* Number of haplotypes; *Hd* Haplotype diversity; *TD* Tajima's D test.

*$P < 0.05$; **$P < 0.01$.

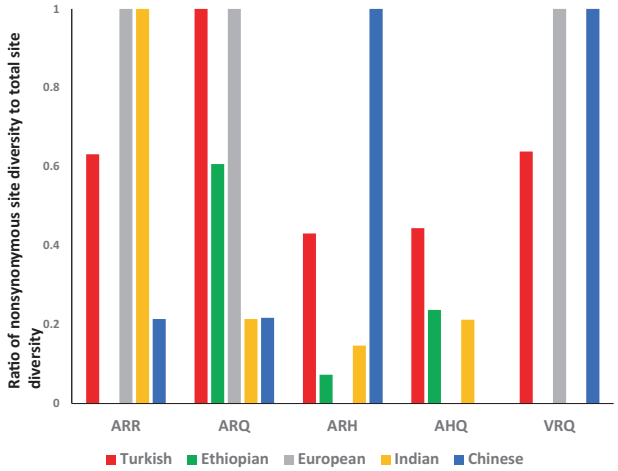

Fig. 1 Comparison of nonsynonymous site nucleotide diversity to total site nucleotide diversity (π) ratio among scrapie associated haplotypes in sheep *PRNP* sequences from different countries. Y-axis shows the ratio of nonsynonymous site pairwise nucleotide diversity (π_{NS}) to nonsynonymous site plus synonymous site pairwise nucleotide diversity. $[\pi_{NS}/(\pi_{NS}+\pi_S)]$. -axis shows the scrapie-associated sheep *PRNP* haplotypes. ARR is the resistant haplotype; VRQ is the susceptible haplotype; ARQ, ARH, and AHQ are associated with little to moderate resistance.

in any sheep population with respect to mouflon and goat, can argue for balancing selection, favoring abundant amino acid changes, together with weak purifying selection on the *PRNP* gene. Our results are partially in agreement with previous reports, where excess replacement changes were observed in sheep *PRNP* (DeSilva et al. 2003; Heaton et al. 2003; Rongyan et al. 2008), and possible balancing selection on *PRNP* was suggested (Slate 2005).

The PRNP story can be more complicated than just a simple classical scrapie association. Although much less frequent, atypical scrapie and different prion strains have been described (Foster and Dickinson 1988; Nonno et al. 2020). Classical scrapie resistant haplotypes (such as ARR) may not be resistant for atypical scrapie (Benestad et al. 2008). *PRNP* genotypes may also have a role in susceptibility to sheep version of bovine spongiform encephalopathy (BSE) (Goldmann et al. 2006; Saunders et al. 2009; Houston et al. 2015). Moreover, PRNP can have diverse pleiotropic physiological roles. In scrapie free sheep populations, a possible selective advantage of postnatal survival, and better survival under harsher environments for the high to moderate susceptibility ARQ allele compared to scrapie resistant (ARR or AHQ) alleles has been suggested (Eglin et al. 2005; Nicholls et al. 2006; Sawalha et al. 2007). *PRNP* can have pleiotropic effects or can be in linkage disequilibrium with genes affecting these traits, at least in some breeds. Moreover, studies suggest involvement of prion protein in several nervous system processes, circadian rhythm, oxidative stress response, development, and immunity (McLennan et al. 2004; Nishida et al. 1999; Cagampang et al. 1999; Katamine et al. 1998; Makzhami et al. 2014; Linden et al. 2008). Protein-protein interaction network (STRING) analyses with sheep, human, and mouse PRNP showed interaction of PRNP with several other proteins that are significantly enriched in diverse biological processes (Supplementary Tables 3–8, Supplementary Fig. 4). Direct or indirect involvement in several biological processes can impose context dependent different selective constraints on the prion protein. A nucleotide change can be advantageous with respect to a phenotype such as disease resistance but can be disadvantageous with respect to another physiological role. That nucleotide change then will not be able increase in frequency and will be kept at a lower frequency in the population or in a specific breed. Therefore, one cannot rule out frequency-dependent selection on the *PRNP* gene. Moreover, most sheep populations are kept as breeds that are bred for specific traits. In that sense, breeds are not one big panmictic populations, but should be considered more like inbred populations. The influence of a potential frequency-dependent selection can be exaggerated in an artificially inbred maintained population as the frequency of harmful alleles can increase by drift.

There are several limitations of our study. Differences in samples size from different populations and breeds may bias population genetic parameter estimates. Study sample size and diversity of world-wide breeds included in the study is not enough to clearly differentiate alternative selection scenarios or quantify the selection coefficients for balancing, frequency dependent, and weak purifying selection. Also, our conclusions with respect to *PRNP* should be tested with other genes and noncoding regions that are both in linkage with *PRNP*, and at an unlinked distance on the sheep chromosome 13. Our findings should be evaluated with respect to possible *PRNP* haplotypes' role in susceptibility to atypical scrapie and BSE in future studies.

We conclude that selection favoring excessive replacement changes are evident in all studied sheep populations and scrapie-associated haplotypes from three continents. Scrapie, at least for the last few hundred years, do not appear to be a major selective force shaping the evolutionary dynamics of *PRNP* evolution. Rather, possible pleiotropic effects with respect to other phenotypes and/or interactions with other genes can be important in the *PRNP* sequence evolution. Moreover, interactions between amino acid residues can be important for prion protein structure and stability (Teferedegn et al. 2020b; Sabate et al. 2015; Terry and Wadsworth 2019). Therefore, adopting selective breeding regimens aiming to eradicate scrapie susceptible alleles can be rather counterproductive if not detrimental for the local sheep breeds adapted for their unique environments.

DATA AVAILABILITY

Data archiving data has been archived with NCBI under the following accession codes. Ethiopian sheep sequences MN834021–MN834117; Turkish sheep sequences JX187517.1–JX187538.1; goat (Capra hircus) sequences MN795374–MN795447. European, Indian, and Chinese PRNP sequences had already been archived in NCBI with following accession codes. European AF180389.1–AF195247.1, AJ000679.1–AJ67988.1, MH934799.1–MH4909.1; Indian DQ013286.1–DQ885794.1; Chinese AB307632.1–AB373810.1, HM639748.1-HM803994.1, HQ197668.1–HQ897614.1, JF310745.1–JF514143.1.

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AUTHOR CONTRIBUTIONS

ET involved in data collection, performed laboratory activities, synthesized and analyzed the data; YY involved in data collection, performed laboratory activities, interpreted results, and edited the manuscript; CU organized the study, interpreted results, and edited the manuscript; ES collected data, formulated and organized the study, synthesized and analyzed the data, wrote the paper. All authors reviewed and approved the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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