RESEARCH ARTİCLE

Prion Protein Gene Polymorphisms in Turkish Native Goat Breeds

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Running Title: *PRNP* polymorphism in Turkish goats.

Abstract

Susceptibility to scrapie in goats is influenced by polymorphisms of the prion protein (*PRNP*) gene. The aim of this study was to identify *PRNP* gene polymorphisms in a total of 356 scrapie disease-free goats from ten Turkish native breeds. Eighteen single nucleotide polymorphisms were detected in the caprine *PRNP* open reading frame. Ten previously described amino acid substitutions (I142M, H143R, N146S N146D, R151H, R154H, P168Q, R211Q, Q222K and P240S) and two novel dimorphisms, G134E and Q163P, were identified. The strongest association between caprine *PRNP* and relative resistance to scrapie disease has been reported previously for polymorphisms at codons 146 (S/D) and 222 (K). In the present study, these three PrP variants were relatively rare with 6.3%. This is the first report on *PRNP* gene variation in Turkish native goat breeds and our knowledge of these polymorphisms will assist goat breeding programs to reduce the risk of scrapie.

Keywords: Scrapie, *PRNP*, polymorphism, Turkish native goat breeds.

Introduction

Goat production is economically and socially important in Turkey. An estimated 500,000 farm households keep goats and goat production contributes directly to the income of nearly three million

people through conversion of natural vegetation into valuable products such as meat, milk, hair (mohair), skins and manure (Gürsoy 2006). All goat breeds farmed in Turkey are well adapted to the marginal lands of Anatolia and contribute to the livelihood of resource-poor farmers living under extremely difficult conditions in the semi-arid areas and highlands of the country (Yilmaz *et al.* 2012).

Scrapie is a fatal, neurodegenerative disease that affects sheep and goats. It is a member of the transmissible spongiform encephalopathies (TSEs) also known as prion diseases that include Creutzfeldt–Jakob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in cattle. In these diseases, a neuronal glycoprotein known as prion protein PrP^{C} (encoded by the *PRNP* gene) is converted into an abnormal protease-resistant isoform (PrP^{Sc}), which accumulates in the central nervous system and lymphoid tissues (Prusiner, 2004). Caprine PrP^{C} contains 256 amino acids, folded into two domains, of which only the C-terminal globular domain (position 102 to 234) is essential for the conversion into pathogenic PrP^{Sc}. There have been no reported scrapie cases in either sheep or goats in Turkey, but the OIE (World Organisation for Animal Health) does not consider Turkey free of disease in accordance with their regulations (OIE, 2010).

In sheep and goats the resistance or susceptibility to scrapie is strongly controlled by polymorphisms of the *PRNP* gene and modulated by the strain of prion disease agent (Goldmann, 2008). It is well established that the ovine allele encoding PrP^{C} with amino acids alanine (A), arginine (R) and arginine (R) at codons 136, 154 and 171 respectively (short: ARR) is associated with a high protection against classical forms of scrapie disease; this association appears to be applicable to scrapie outbreaks worldwide. In contrast, PrP variants VRQ or ARQ are associated with susceptibility and this is more agent strain dependent (Hunter 1997). Genetic association of *PRNP* with atypical forms of scrapie disease is slightly more complicated and involves different amino acid substitutions such as L141F (Benestad *et al.* 2008). With EU Decision 2003/100/ EC (EU, 2003), each member state in the European Community has introduced breeding strategies to increase the frequency of the resistance-associated PrP variant ARR in their sheep populations.

PRNP genotyping of all native Turkish sheep breeds let to the identification of a considerable number of polymorphisms and helped to evaluate the genetic risk for both classical and atypical scrapie (Meydan *et al.* 2012, 2013a, 2013b).

To date, worldwide more than 40 amino acid substitutions have been described in the caprine *PRNP* open-reading frame but only a few of them - I142M, H143R, G145D, N146S/D, Q211R and Q222K - have been proven to be associated with resistance to scrapie disease. Several case/control studies performed in Italy (Acutis *et al.* 2006; Vaccari *et al.* 2006) and France (Barillet *et al.* 2009) demonstrated a high protective effect of the K222 variant against classical scrapie. The codon 146 variants S146 and D146 have been similarly associated with scrapie resistance in Cypriot herds (Papasavva-Stylianou *et al.* 2011, Ortiz-Pelaez *et al.* 2014). Modulation of classical scrapie disease by amino acid substitutions in codons 142, 143, 145, 154 and 211 has been shown by studies from the UK, Italy, France, and Greece (Goldmann *et al.* 1996; Billinis *et al.* 2002, Vaccari *et al.* 2006, Barillet *et al.* 2009, González *et al.* 2009; Bouzalas *et al.* 2010, Colussi *et al.* 2010; Maestrale *et al.* 2015). The strength of the K222 association with scrapie and BSE resistance was confirmed in experimental challenges (Acutis et al, 2012, White et al., 2012, Aguilar-Calvo *et al.* 2014, 2015). It is therefore likely that similar to the ovine ARR allele, in goats a universal, relative strain-independent protection from classical scrapie is conferred by K222, S146 and D146.

As yet there has been no study on the caprine *PRNP* gene variation in Turkey. Although there have been no officially reported cases of scrapie in Turkish goats, information regarding *PRNP* gene polymorphisms in Turkish goat breeds may assist future goat breeding programs to reduce the possible risk of scrapie. Additionally, the combination of old breed types and substantial environmental adaptations raises particular interest to explore genetic variation in the *PRNP* gene. The objective of this study was to genotype ten Turkish goat breeds in order to determine polymorphisms of the *PRNP* open reading frame and evaluate their theoretical genetic susceptibility to scrapie.

Materials and Methods

Sampling and DNA extraction

A total of 356 unrelated healthy goats, 2-5 years old, were randomly sampled from ten breeds (for details see Table 1). Blood samples were collected from the jugular vein into EDTA vacutainers and stored at -20°C until genomic DNA extraction, which was carried out using a salting-out method (Miller *et al.* 1988). All procedures were approved by the Animal Experimentations Local Ethics Board at Ankara University.

PCR assay and DNA sequencing

A 489bp fragment (*PRNP* codons 103-249) was amplified by PCR. The amplification reactions were prepared in a final volume of 50 µl containing as follows; 1 × PCR buffer, 0.8 mM dNTPs, 1.5 units *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 20 pmol of forward TCAAGGTGGTAGCCACAGTCAGT and reverse CTATCCTACTATGAGAAAAATGAG primers (Billinis *et al.* 2002) and approx. 1µg genomic DNA. Amplification was performed using an initial denaturation of 5 minutes at 95°C, followed by 40 cycles of 60 seconds at 95°C, 60 seconds at 60°C and 90 seconds at 72°C and a final extension of 7 minutes at 72°C. PCR products resolved by electrophoresis on 2% agarose gels. After gel electrophoresis, the amplicons were purified using a Qiamp Mini Kit (QIAGEN, Valencia, CA, USA). The purified samples were sequenced by Big Dye Terminator chemistry on an ABI 3100 Avant Automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Haplotypes and pairwise F_{ST} values were calculated with DNASP (Librado and Rozas 2009) and ARLEQUIN (Excoffier *et al.*, 2006), an unrooted neighbor-joining (NJ) dendrogram was generated with SPLITS TREE4 (Huson and Bryant 2006).

Results

We found twelve amino acid substitutions: G134E, I142M, H143R, N146D, N146S, R151H, R154H, Q163P, P168Q, R211Q, Q222K and P240S (Figure 1). Two of them have not been reported previously: in codon 134, a substitution of GGA→GAA leading to amino acid change

glycine (G) → glutamic acid (E) and in codon 163 substitution CAA→CCA leading to amino acid change glutamine (Q) → proline (P). These novel polymorphisms were detected in Norduz and Angora goats, respectively. In addition six silent mutations at codons 119 (t→c), 133 (g→c), 138 (t→c), 141 (t→c), 179 (g→t) and 219 (c→t) were observed, only 119, 133 and 141 were novel. All amino acid substitutions and silent mutations, and their distributions in Turkish native goat breeds are shown in Table 2.

Genotypes Seq1/Seq1, Seq2/Seq2 or Seq1/Seq2 (for allelic sequences see Table 3) were found in 82% of all goats. Seq1 is considered to be the caprine wild-type PrP^C sequence and it was found here as predominant variant with a frequency of 0.56 (402/712). Differing only in position 240 (P→S) was Seq2 with a frequency of 0.26; this allele is often regarded as the PrP ruminant archetype as it is most similar to PrP of other species. The remaining 64 (18%) goats gave rise to 15 protein sequences, eight with P240 and seven with S240 confirming that the codon 240 dimorphism lies at the root of PrP protein divergence (Seq3-Seq13, Table 3).

The new dimorphisms, G134E and P163Q were found in more than one animal in heterozygous genotypes (Figure 2). Four amino acid dimorphisms were detected in only one breed each: E134 in Norduz, H151 in Damascus, P163 in Angora and K222 in the non-indigenous Saanen. While the resistance-associated K222 variant was not found in the native breeds, other resistant PrP variants with polymorphisms such as S146 and D146 were present in two and four out of nine indigenous breeds, respectively; with average frequencies of 0.17 (S146) and 0.04 (D146) in those breeds or a combined frequency of 0.059 for all breeds. The M142 and Q211 variants were only detected in Akkeçi and Saanen goats with combined breed frequencies of 0.534 and 0.17, respectively. Based on pairwise F_{ST} values calculated from the frequencies of observed *PRNP* haplotypes, a phylogenetic tree was constructed as a representation of the genetic relationship among the Turkish goat breeds at the *PRNP* locus (Figure 3).

Discussion

Scrapie is a fatal disease for which no treatment is available. It can have devastating consequences for small farm holdings and the only long term prevention method is differential selection against susceptible and for resistant *PRNP* genotypes. In recent years, reports of caprine *PRNP* polymorphisms and case-control studies in different European countries have revealed the value that a genetic approach could have to minimize disease risk. In Turkey, scrapie control breeding programs have not been established for sheep and goats because there have not been any confirmed scrapie cases. Although all Turkish native sheep breeds have been *PRNP* genotyped to evaluate the genetic risk for both classical and atypical scrapie (Meydan *et al.* 2012, 2013a, 2013b), this is the first study to *PRNP* genotype all Turkish native goat breeds.

A total of seventeen different PrP^C protein sequences were deduced from the DNA sequences amongst which the wildtype PrP^C is the most common in Turkish goats and also the predominant allele in all breeds except Akkeçi goats (Table 3). Corbiere *et al.* (2013) reported that the S240P polymorphism appears to have no direct impact on scrapie disease risk, which is important as this S240P dimorphism is the only amino acid change that occurs in various combinations with the other amino acid substitutions. The remaining 18% of the goats carried at least one other PrP^C variant and this frequency is not dissimilar to European populations (Goldmann, 2008). Discounting the very common polymorphism S240P, on average one amino acid substitution was found for every 36 genotypes revealing that Turkish goats are at least as genetically varied in this focus as Turkish sheep, for which we genotyped on average 52 animals / substitution (Meydan et al., 2013a).

Ten out of the twelve amino acid substitutions observed here have previously been described in goats from several countries including Mediterranean countries (Greece, Cyprus, Spain, Morocco, Italy) whereas two were novel (G134E and Q163P). A priori predictions of the effect that these amino acid changes may have on scrapie susceptibility are not yet possible, but neither substitution is conservative, so that structural changes may be likely for these variants. It should be noted that the codon positions 134 and 163 fall into a region of PrP^C (Figure 1 A, B) in which there

are fifteen known disease-associated amino acid changes (Figure1 C) and 14/15 are conferring a degree of resistance (Goldmann, 2008).

S146 (Seq6) and D146 (Seq7) have both been associated with strong protection from classical scrapie in Cypriot goats (Papasavva-Stylianou *et al.* 2011). With a combined frequency of 5.9% and representation in five out of 10 breeds they could become target alleles for resistance breeding. Indeed, S146 was the most frequent PrP variant (18/64) in Damascus goats, suggesting that this resistance-associated polymorphism may be maintained in the Damascus-related breeds independent of the presence of scrapie. K222 (Seq13) is another strongly protective PrP^C variant; only Turkish Saanen goats carried the K222 with a frequency of 3.4%, which is similar to other European populations. Further surveys will be needed to confirm this estimate and whether it is truly absent from the indigenous breeds.

From the point of view of future breeding programs for the eradication of scrapie the most valuable polymorphisms to have found in this study are S146, D146 and K222 (K). This evaluation is based of course on the assumption that if prion strains were circulating in Turkey, that they would be similar to European prion strains and therefore *PRNP* genetics would follow the same rules. As there are no detected scrapie cases in Turkey this remains speculative, but prion disease genetics in sheep has shown strongly resistance-associated PrP^C variants appear to be universally effective.

In this study, the combined frequency of these three resistance-associated PrP variants in all Turkish goat breeds was approximately 6.3%, which is a low starting point for breeding programs. However when only the five breeds which carried these alleles are considered the combined frequency of 18.8% (39/208) represents a realistic foundation for breeding strategies. Additionally it should be noted that M142, H154 and Q211 also have partially protective effects on classical scrapie and they contribute another 6.5% of the total surveyed goats or 20.2% of the six breeds in which the alleles were found.

While Damascus goats had the largest proportion of resistance-associated PrP variants, others, like Imroz and Malta breeds had no goats in this category (Table 2). The non-indigenous

Saanen breed, which is reared in southern part of Turkey, appeared to be the most variable breed in Turkey with seven variants and cross-breeding with indigenous breeds may be advantages from the scrapie perspective. As expected from their breed history, Akkeçi and Saanen goats shared many haplotypes (Table 2); their genetic similarity can be seen on the neighbor joining (NJ) dendrogram (Figure 3). The most resistant Turkish goat breed may be Damascus, further research will be needed to confirm this finding.

The earliest evidence of goat domestication was found in the central Anatolia (modern day Turkey) (Zeder 2008). Thus, it is likely that some of the Turkish native goat breeds of today are some of the oldest living descendants of their first domesticated ancestors, and Anatolian (Turkish) native breeds may be special in maintaining very valuable genetic diversity. Our caprine *PRNP* gene survey supports this view by showing that Turkish breeds are genetically diverse and that they share a considerable number of *PRNP* polymorphisms with the breeds of the world.

Conclusion

This study is first report to identify the *PRNP* gene polymorphism in Turkish native goat breeds. Two novel amino acid dimorphisms, G134E and Q163P were observed and dimorphisms associated with high resistance to scrapie were present, amongst them S146, D146 and K222. This result is encouraging with regard to goat breeding programs to reduce the risk of scrapie in Turkey.

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Competing interests

The authors declare that they have no competing interests.

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Table 1. Sampling localities, sample size (n) and sex of Turkish native goat breeds

Breed	Abbreviation	Sampling Location	n	Sex
Akkeçi	AKK	Ankara	16	13♀ + 3♂
Ankara (Angora)	ANK	Çankırı	15	12♀ + 3♂
		Ankara	23	19♀ + 4♂
Gökçeada (Imroz)	GOK	Çanakkale	20	16♀ + 4♂
Halep (Damascus)	HLP	Şanlıurfa	32	27♀ + 5♂
Honamlı	HNM	Antalya	42	36♀ + 6♂
Kıl (Hair goat)	KIL	Konya	25	20♀ + 5♂
		Ankara	25	20♀ + 5♂
Malta	MLT	Edirne	28	23♀ + 5♂
Norduz	NRD	Van	46	40♀ + 6♂
Kilis	KLS	Gaziantep	20	16♀ + 4♂
		Kilis	20	16♀ + 4♂
Saanen	SNN	İzmir	44	38♀ + 6♂
Total			356	296♀ + 60♂

Table 2. Number of various *PRNP* gene polymorphisms and their distributions in Turkish native goat breeds

Variation			Breeds									
DNA[†]	Change	Protein	AKK	ANK	GOK	HLP	HNM	KIL	MLT	NRD	KLS	SNN	Total
357T → C	GCT/GCC	None*					2						2
399G → C	CTG/CTC	None*		1									1
401G → A	GGA/GAA	G134E*								2			2
414T → C	AGT/AGC	None	5	11	4	4	7	10	7	6	12	8	74
423T → C	CTT/CTC	None*		1									1
426A → G	ATA/ATG	I142M	10									14	24
428A → G	CAT/CGT	H143R					10			2			12
436A → G	AAT/GAT	N146D					2	4			6	2	14
437A → G	AAT/AGT	N146S				14		8					22
452G → A	CGT/CAT	R151H				2							2
461G → A	CGT/CAT	R154H		2		6	2			2			12
488A → C	CAA/CCA	Q163P*		2									2
503C → A	CCA/CAA	P168Q		4			4		4	2		2	16
537G →T	GTG/GTT	None						5					5
632G → A	CGA/CAA	R211Q	4									4	8
657C → T	ACC/ACT	None								3			3
664C → A	CAG/AAG	Q222K										3	3

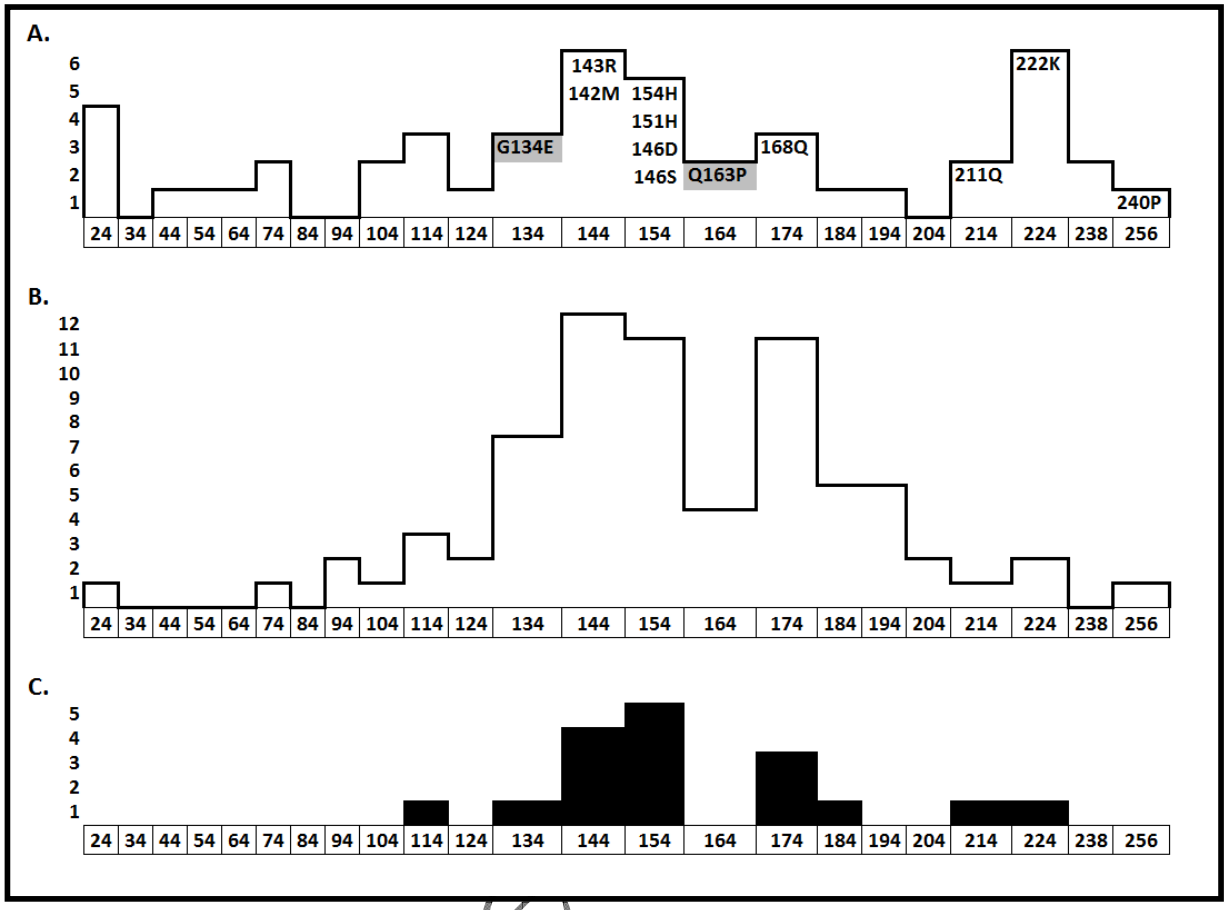
Numbers are representing the number of animals with those polymorphisms, either homozygous or heterozygous. [†]DNA position are given to the nucleotide positions of NM_001314247. *Polymorphisms not previously reported, these were confirmed by sequencing twice and in both directions on DNA from different PCR reactions. AKK, Akkeçi; ANK, Angora; GOK, Imroz; HLP, Damascus; HNM, Honamlı; KIL, Hair goat; MLT, Malta; NRD, Norduz; KLS, Kilis; SNN, Saanen.

Table 3. Deduced protein variants of *PRNP* gene and their frequencies in Turkish native goat breeds.

	PRNP codon											AKK	ANK	GOK	HLP	HNM	KIL	MLT	NRD	KLS	SNN	TOTAL
Seq	134	142	143	146	151	154	163	168	211	222	240											
1	G	I	H	N	R	R	Q	P	R	Q	P	*0.125*	*0.632*	*0.700*	*0.436*	*0.500*	*0.580*	*0.606*	*0.672*	*0.675*	*0.500*	*0.564*
2	-	-	-	-	-	-	-	-	-	-	S	*0.375*	*0.261*	*0.300*	*0.158*	*0.285*	*0.280*	*0.322*	*0.240*	*0.250*	*0.216*	*0.260*
3	E	-	-	-	-	-	-	-	-	-	P	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0.022*	*0*	*0*	*0.003*
4	-	M	-	-	-	-	-	-	-	-	P/S	*0.375*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0.159*	*0.037*
5	-	-	R	-	-	-	-	-	-	-	P/S	*0*	*0*	*0*	*0*	*0.119*	*0*	*0*	*0.022*	*0*	*0*	*0.017*
6	-	-	-	S	-	-	-	-	-	-	P/S	*0*	*0*	*0*	*0.281*	*0*	*0.100*	*0*	*0*	*0*	*0*	*0.039*
7	-	-	-	D	-	-	-	-	-	-	P	*0*	*0*	*0*	*0*	*0.024*	*0.040*	*0*	*0*	*0.075*	*0.023*	*0.020*
8	-	-	-	-	H	-	-	-	-	-	S	*0*	*0*	*0*	*0.031*	*0*	*0*	*0*	*0*	*0*	*0*	*0.003*
9	-	-	-	-	-	H	-	-	-	-	P/S	*0*	*0.027*	*0*	*0.094*	*0.024*	*0*	*0*	*0.022*	*0*	*0*	*0.017*
10	-	-	-	-	-	-	P	-	-	-	P	*0*	*0.027*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0.003*
11	-	-	-	-	-	-	-	Q	-	-	P	*0*	*0.053*	*0*	*0*	*0.048*	*0*	*0.072*	*0.022*	*0*	*0.023*	*0.022*
12	-	-	-	-	-	-	-	-	Q	-	S	*0.125*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0.045*	*0.011*
13	-	-	-	-	-	-	-	-	-	K	S	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0.034*	*0.004*

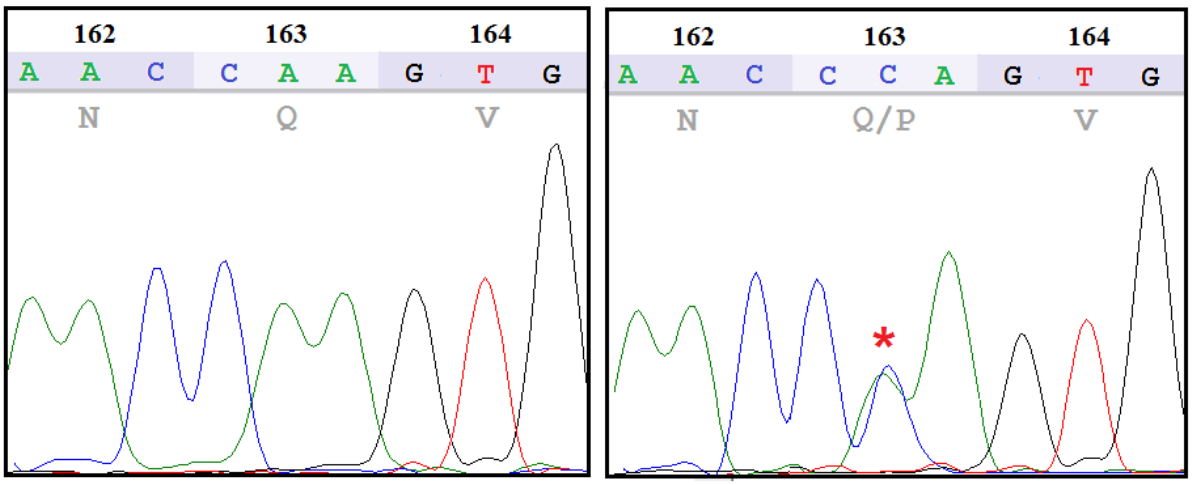
AKK, Akkeçi; ANK, Angora; GOK, Imroz; HLP, Damascus; HNM, Honamlı; KIL, Hair goat; MLT, Malta; NRD, Norduz; KLS, Kilis; SNN, Saanen.

Figure 1. Histograms showing the number of amino acid substitutions (polymorphisms) on *PRNP*.



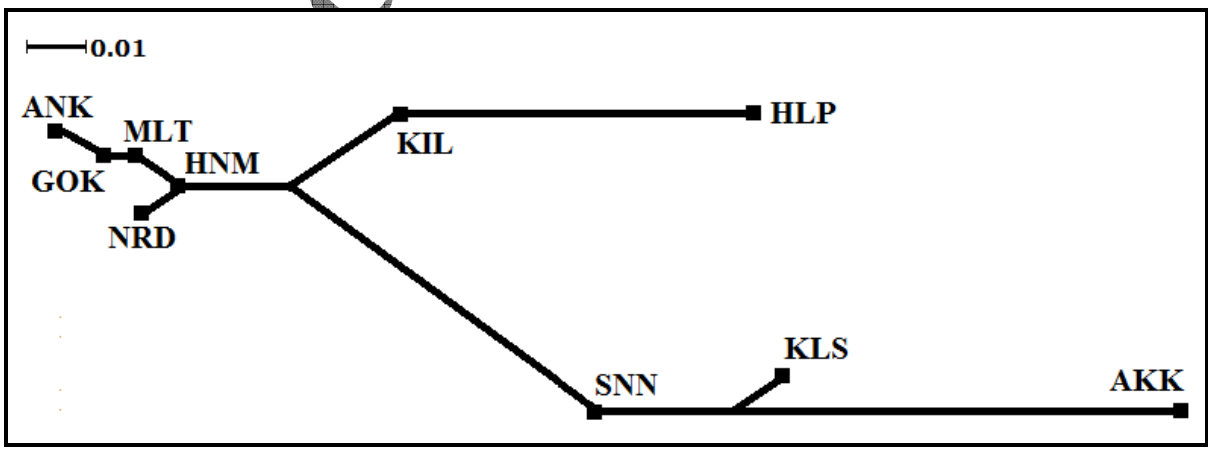
Sequence is divided into consecutive blocks of 10 amino acids with the exception of the first block (amino acids 1–24, signal peptide) and last block (239–256, signal peptide). (A) Distribution of 47 caprine polymorphisms including the substitutions described in this study; 134E and 163P are novel polymorphisms. (B) Shown for comparison is the distribution of 71 ovine polymorphisms. (C) Distribution of 17 ovine and caprine polymorphisms that have been shown to be associated with scrapie disease susceptibility and resistance in worldwide case/control studies.

Figure 2. Sequence chromatogram for codon 163 of *PRNP* in homozygote and heterozygote animals.



Left panel: homozygous genotype 163QQ deduced from sequence CAA; right panel: heterozygous genotype 163 (QP) deduced from sequence C(C+A)A representing CAA (Q) and CCA (P). Red star indicates the polymorphic site. Q=glutamine, P=proline.

Figure 3. Neighbor joining (NJ) dendrogram for Turkish native goat breeds based on *PRNP* haplotypes.



AKK, Akkeçi; ANK, Angora; GOK, Imroz; HLP, Damascus; HNM, Honamlı; KIL, Hair goat; MLT, Malta; NRD, Norduz; KLS, Kilis; SNN, Saanen.