SURVEILLANCE OF PRION PROTEIN (PrP) GENE POLYMORPHISMS IN TURKISH NATIVE SHEEP BREEDS

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ABSTRACT

SURVEILLANCE OF PRION PROTEIN (PrP) GENE POLYMORPHISMS IN TURKISH NATIVE SHEEP BREEDS

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Scrapie is an infectious fatal disease of sheep and goats which affects the central nervous system. In the present study, samples of 14 native Turkish sheep breeds (n=655) were analyzed with respect to their polymorphisms of *PrP* gene (at codons: 136, 141, 154 and 171) and their classical and atypical scrapie risk levels were identified.

Turkish sheep are found to have the highest PrP genetic variability with 13 classical scrapie alleles and 14 atypical scrapie alleles compared to all previous studies. Classical scrapie-susceptible and wild-type ARQ allele was found as the most frequent allele in Turkish sheep examined. The most classical scrapie-susceptible allele, VRQ was detected at low frequencies in 5 of the breeds (Çine Çaparı, Dağlıç, Kıvırcık, Karayaka and Gökçeada). One novel allele ($TL_{141}HQ$) was observed in Sakız breed for the first time in this study.

It was found that most of the classical scrapie genotypes belong to R3 risk group, whereas atypical scrapie genotypes belonging to zero (0) and one (1) risk groups were frequently seen in sheep analyzed. In other words, Turkish sheep is found to have intermediate risk of classical scrapie and low atypical scrapie risk, in general.

The data from the current study may help to establish a breeding program for classical scrapie control in Turkey and will be beneficial for both the animal and public health in the country. In addition, the outcomes of the study will fill the gap which is present in the geographic distribution data of *PrP* gene polymorphisms in Eurasia.

Keywords: Turkish native sheep breeds, classical scrapie, atypical scrapie, prion protein gene, polymorphism

ÖZ

TÜRKİYE YERLİ KOYUN IRKLARINDA PRİON PROTEİN (PrP) GENİ POLİMORFİZMLERİNİN TARANMASI

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Skrapi, koyun ve keçilerin merkezi sinir sistemini etkileyen bulaşıcı ve ölümcül bir hastalıktır. Mevcut çalışmada, 14 yerli Türk Koyun ırkının (n=655) örnekleri, *PrP* gen polimorfizmleri (136, 141, 154 ve 171. kodonlardaki) baz alınarak incelenmiş ve klasik ve atipik skrapi risk seviyeleri belirlenmiştir.

Önceki çalışmalar ile karşılaştırıldığında, Türkiye'deki koyunların 13 adet klasik skrapi alleli ve 14 adet atipik skrapi alleli ile en yüksek PrP genetik çeşitliliğine sahip oldukları bulunmuştur. Hem klasik skrapiye yatkın hem de yabani allel olan ARQ alleli, Türkiye koyun ırklarında en yaygın allel olarak bulunmuştur. Klasik skrapiye en yatkın allel olan VRQ alleli ise düşük frekanslarda 5 yerli koyun ırkında (Çine Çaparı, Dağlıç, Kıvırcık, Karayaka ve Gökçeada) tespit edilmiştir. Yeni ve daha önce rastlanmamış bir allel ($TL_{141}HQ$) Sakız ırkında ilk defa sunulan bu çalışmada saptanmıştır.

Türkiye yerli koyun ırklarında, klasik skrapi ile ilişkili genotiplerin çoğu R3 risk grubuna dahil olurken sıfır (0) ve bir (1) risk gruplarına dahil olan atipik skrapi ile ilişkili genotipler en yaygın olarak görülmüştür. Başka bir deyişle, genel olarak, Türkiye

koyunlarının orta seviye klasik skrapi riskine ve düşük atipik skrapi riskine sahip oldukları bulunmuştur.

Sunulan çalışmada elde edilen veriler, Türkiye'de klasik skrapi kontrolü için bir yetiştirme programının oluşturulmasına yardım edebilir ve ülkedeki hem hayvan hem de halkın sağlığı için faydalı olacaktır. Buna ek olarak, mevcut çalışmanın sonuçları Avrasya'daki *PrP* gen polimorfizm verilerinin coğrafi dağılımındaki boşluğu dolduracaktır.

Anahtar kelimeler: Türkiye yerli koyun ırkları, skrapi, prion protein geni, polimorfizm, evrimsel etmenler

To My Grandmother and Grandfather,

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

°C : Degrees Celsius

µl : Microliter

Arlequin: An Integrated Software Package for Population Genetics Data Analysis

bp : Base Pair

dNTP: Deoxynucleotide Triphosphate

dH_2O : Distilled Water

DNA : Deoxyribonucleic Acid

EDTA : Ethylene Diamine Tetra Acetic Acid

e.g: For example

HPG: Haplogroup

K_3EDTA: Potassium EDTA

MEGA : Molecular Evolutionary Genetics Analysis

MgCl2 : Magnesium Chloride

mM: Millimolar

mtDNA : Mitochondrial DNA

NSP: National Scrapie Plan

NJ: Neighbor Joining

Nor98: Atypical form of scrapie

NTSYS: Numerical Taxonomy and Multivariate Analysis System

ORF: Open Reading Frame

PCR: Polymerase Chain Reaction

pH : Potential of Hydrogen

PHYLIP: Phylogeny Inference Package Software

PrP : Protein related prion

PrP^{C}: Normal cellular prion protein

PrP^{Sc}: Pathogenic prion protein

rpm : Rotations per Minute

SNP: Single Nucleotide Polymorphism

Taq : Thermus aquaticus

TBE: Tris Borate EDTA

TSE: Transmissible Spongiform Encephalopathies

TUBITAK: The Scientific and Technological Research Council of Turkey

TURKHAYGEN-I: *In Vitro* Conservation and Preliminary Molecular Identification of Some Turkish Domestic Animal Genetic Resources-I (TUBITAK Project No: 106G005)

UV: Ultra Violet

V: Volt

CHAPTER 1

INTRODUCTION

Scrapie is a neurodegenerative infectious fatal disease of sheep, goats and moufflons (Jeffrey and González, 2007) and it belongs to the family of prion diseases or transmissible spongiform encephalopathies (TSEs) as will be explained in detail in the following sections. The disease is called "scrapie" since infected sheep and goats often rub on objects and scrape off their wool or hair. Scrapie is characterized by the deposition of pathogenic isoform of prion protein (PrP), which is oftenly symbolized as PrP^{Sc}, in the central nervous system (CNS) (Acin *et al.,* 2004).

1. 1 Prion Protein (PrP) and Prion Diseases

Prion proteins are synthesized both in the central nervous system and in a variety of cells (e.g., heart, kidney, lung, pancreas, testis, platelets and leukocytes) in living animals (Detweiler and Baylis, 2003). The function of the normal prion proteins are not known, however they might have been playing a role in the immune system (Isaacs *et al.,* 2006).

The term "Prion" was used by Prusiner (1982) for the first time indicating infectious protein particles (Imran and Mahmood, 2011) closely associated with prion disease infectivity and may be the causative agent of prion diseases. The prion protein exists in two forms encoded by the *PrP* gene (Prusiner, 1991). The PrP^{C} form of prion protein (PrP) indicates the normal cellular prion protein, whereas the PrP^{Sc} form is pathogenic, abnormal form associated with prion diseases. The difference between PrP^{Sc} and PrP^{C} is in their biochemical properties (Sipos *et al.,* 2002). PrP^{C} is alpha-helical, whereas PrP^{Sc} is at least 40% beta-pleated sheet (Pan *et al.,* 1993; Safar *et al.,* 1998). In addition, PrP^{C}

is soluble in non-denaturing detergents, while PrP^{Sc} is insoluble. Also, PrP^{C} can be completely degraded by proteases, but PrP^{Sc} has a relative resistance to proteases. In Figure 1. 1 the three-dimensional structures of PrP^{Sc} and PrP^{C} are shown (Huang *et al.,* 1995).

According to prion hypothesis, when the normal cellular prion protein (PrP^{C}) changes into an isoform (PrP^{Sc}) of which β-sheets have been increased (Figure 1. 1) the disease emerges. In other words, prions cause a geometrical conformation change in prion protein (PrP) resulting in the occurrence of prion diseases (Billinis *et al.,* 2004). During the disease, the accumulation of abnormal PrP^{Sc} causes nervous system dysfunction and eventually, it causes the death of infected individuals (Detwiler, 1992).

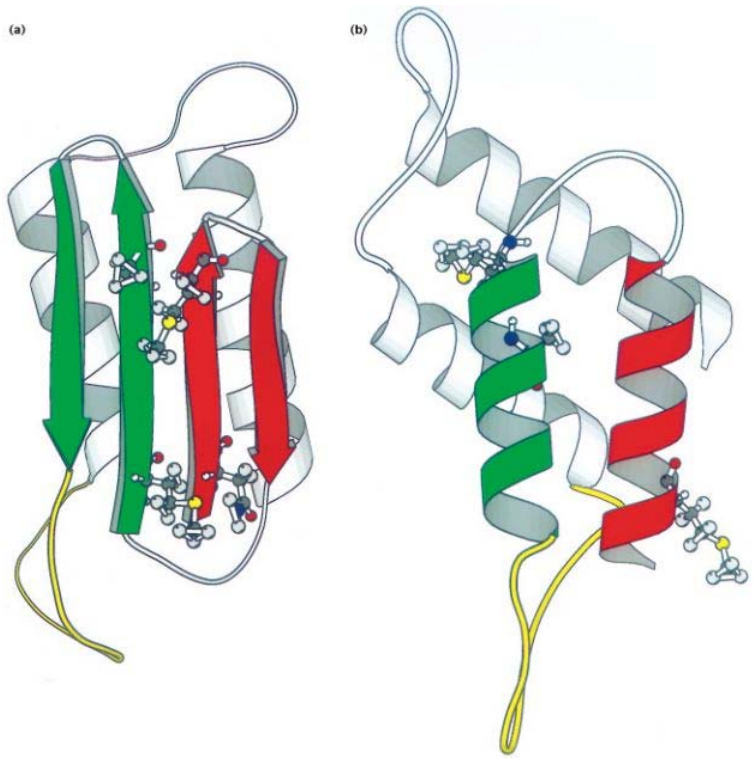


Figure 1. 1 Three-dimensional structures of two forms of prion protein (PrP). a) The proposed three-dimensional structure of PrP^{Sc}. b) The three-dimensional structure of PrP^{C} (Huang *et al.,* 1995).

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are a group of rapidly progressive, invariably fatal, neurodegenerative diseases that affect both humans and animals. Most prion diseases are characterized by a long incubation period, neuronal loss and loss of motor control (Collinge, 2001). They include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) or "mad cow" disease in cattle, chronic wasting disease (CWD) in deer and elk, transmissible mink encephalopathy (TME) in mink, TSE in non-human primates (NHP) in lemurs, feline spongiform encephalopathy (FSE) in cats, exotic ungulate spongiform encephalopathy (EUE) in zoo ruminants and fatal diseases including Creutzfeld-Jacob disease (CJD) in human (Imran and Mahmood, 2011). Up to date, versions of prion diseases in humans and some animal species were given in Table 1. 1.

Table 1. 1 Prion diseases in humans and some animal species (Imran and Mahmood, 2011).

SPECIES	PRION DISEASE
Sheep, Goats	Scrapie
Cattle	Bovine Spongiform Encephalopathy (BSE or "Mad Cow" disease)
Cervids	Chronic Wasting Disease (CWD)
Cats	Feline Spongiform Encephalopathy (FSE)
Mink	Transmissible Mink Encephalopathy (TME)
Lemurs	TSE in non-human primates (NHP)
Nyala, Kudu	Exotic Ungulate Spongiform Encephalopathy (EUE)

Table 1. 1 Continued

SPECIES	PRION DISEASE
Human	Kuru iatrogenic Creutzfeldt–Jakob disease (iCJD) variant Creutzfeldt-Jacob disease (vCJD) familial Creutzfeldt–Jakob disease (fCJD) sporadic Creutzfeldt–Jakob disease (sCJD) Gerstmann-Sträussler-Scheinker syndrome (GSS) Fatal Familial Insomnia (FFI)

Prion diseases (TSEs) are developed not only spontaneously (80%) but also as a result of mutations in *PrP* gene (15%) and infection (5%) (McKintosh *et al.,* 2003).

1. 2 *PrP* Gene

Although the basic gene structure of *PrP* genes in all species are similar, their location and lengths of exons and introns are different as presented in Table 1. 2 (Tranulis, 2002). For instance, human *PrP* gene is located on the short arm of chromosome 20, while ovine, caprine and bovine *PrP* gene is located on chromosome 13 (Castiglioni *et al.,* 1998). In all of these species, *PrP* gene contains two introns and three exons (two non-coding and one coding) with the open reading frame (ORF) located on exon three (Tranulis, 2002; Goldmann, 2008).

Table 1. 2 Comparative lengths (in base pairs) of exons, introns and ORFs in *PrP* gene of some species. Table adapted from the Final TUBITAK Project Report with the Full Name: Surveillance of prion protein gene polymorphisms in Pakistani and Turkish native sheep breeds (TUBITAK –MOST – Project no: 108O708).

Species	Chromosome	Exon 1	Exon 2	Exon 3	ORF	Intron 1	Intron 2
Sheep	13	52	98	4028	768	2421	14031
Goat	13	52	98	4019	771	2430	14020
Cattle	13	53	98	4091	795	2436	13552
Human	20	134	99	2354	762	2622	9975

The ovine *PrP* gene, which encodes for the prion protein, is localized on the 13th chromosome, having three exons of 52, 98 and 4028 base pairs (bp) and two introns of 2421 and 14031 bp. Its 768 bp long open reading frame (ORF) is located on exon three coding for a protein product of 256 amino acids (Goldmann *et al.,* 1990; Lee *et al.,* 1998). The genetic structure of the *PrP* gene in sheep is presented in Figure 1. 2.

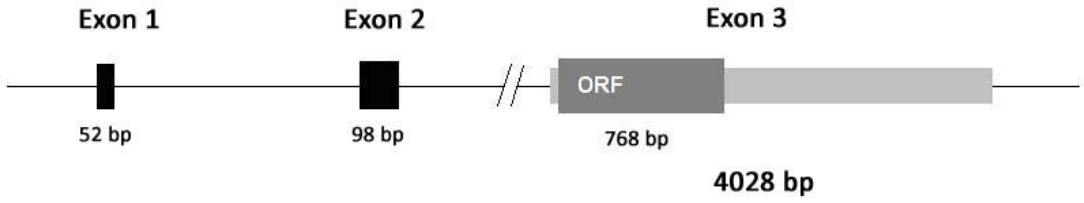


Figure 1. 2 The genetic structure of the ovine *PrP* gene (ORF: Open Reading Frame). Figure adapted from the Final TUBITAK Project Report with the Full Name: Surveillance of prion protein gene polymorphisms in Pakistani and Turkish native sheep breeds (TUBITAK –MOST – Project no: 108O708).

Approximately 50 single nucleotide polymorhisms (SNPs) have been observed at 35 different codons in exon three of the sheep *PrP* gene (Alvarez *et al.,* 2011). However, as will be explained below (Section 1.3.1.3), the polymorhisms at codons 136, 154 and 171 have been determined to be related with the disease scrapie; therefore, polymorhisms at these codons became important for sheep industry, also for animal and human health.

1. 3 Prion Diseases in Sheep

1. 3. 1 Classical Scrapie

1. 3. 1. 1 Epidemology, Transmission and Clinical Signs

Scrapie is a degenerative and 100% fatal disease with a long incubation period (2-5 years), affecting the central nervous system of sheep and goats. The classical scrapie is the oldest prion disease (Goldmann *et al.,* 1990, Benestad *et al.*, 2003) which is known in Europe approximetaly for 280 years (McGowan, 1922) and it also spread to many other countries of the world (Detwiler and Baylis, 2003). The disease was reported for the first time in United Kingdom in 1732 (Thompson and Gasser, 2008) followed by many countries throughout the world. Australia and New Zealand are the only two countries which are now considered to be free of classical scrapie, however scrapie cases have also been found in these countries before but the disease have successfully eradicated (Hunter *et al.,* 1998; Bossers *et al.,* 1999). Table 1. 3 is constituted to show the first time that scrapie disease has been observed in different countries by years.

Table 1. 3 Some countries and years that scrapie was reported in for the first time. Table adapted from the Final TUBITAK Project Report with the Full Name: Surveillance of prion protein gene polymorphisms in Pakistani and Turkish native sheep breeds (TUBITAK –MOST – Project no: 108O708).

COUNTRY	YEAR	COUNTRY	YEAR
United Kingdom	1732	**Norway**	1958-1959
Canada	1938	**South America**	1964-1972
USA	1947	**Colombia**	1968-1971
Australia	1952	**Kenya**	1970
New Zelland	1952-1954	**Brazil**	1977

It is thought that the reason for the intercontinental distribution of scrapie, after it is reported in United Kingdom and many other European countries, may be the worldwide spread of some sheep breeds such as merino sheep that had an economic significance especially during World War II (Detwiler and Baylis, 2003, Detwiler, 1992). Figure 1. 3 represents the spread of classical scrapie from United Kingdom to many other countries throughout the world between 1938 and 1977 (Detwiler and Baylis, 2003).

Figıre 1. 3 The distribution of classical scrapie between 1938 and 1977 (Detwiler and Baylis, 2003).

Today it is considered that scrapie can be transmitted from dams to the offspring vertically. Besides, if the environment is contaminated by the infected fetal membranes, scrapie can be transmitted from ewe to lamb and also among adults (Healy *et al.,* 2004). As a result, susceptible animals will be infected horizontally (Dexter *et al.,* 2009). Moreover, the contact between the infected herds is also considered to be effective in the transmission of classical scrapie (Philippe *et al.,* 2005).

The incubation period of the disease is between 2-5 years and it is rarely observed in lambs younger than age 1 (Dickinson *et al.,* 1976). Infected prions enter the animal's body through both the wounds in the skin and the intestine and causes a conformational change in the cellular prion protein (van Keulen *et al.,* 2000).

The clinical signs of the disease are not constant in all infected sheep and vary depending on the genotype of the animal. However, the reduction of yield loss, weight loss, behavioral changes, itching and rubbing, wool pulling, biting at legs or sides, head pressing, lip smacking, blindness, loss of coordination and tremor are some common symptoms of the disease (Foster *et al.,* 2001; Redman *et al.,* 2002). Figure 1. 4 shows some examples of scrapie infected sheep.

Figure 1. 4 Sheep with scrapie (http://www.teara.govt.nz/en/diseases-of-sheep-cattle-and-deer/4/4/1; http://en.wikipedia.org/wiki/Scrapie)

1. 3. 1. 2 The significance of Classical Scrapie

The disease scrapie leads to the reduction of yield loss followed by death of individuals and it causes economical losses. However, it was never regarded as a risk factor for human health. Besides, there is not a scientific evidence that scrapie is a direct threat to human health, there is strong evidence that the mad cow disease (BSE) which emerged

in 1986 is in connection with the variant Creutzfeld-Jacob disease (vCJD), which is fatal in humans (Bruce *et al.,* 1997). Moreover, in 1996, it is experimentally suggested that sheep can also be infected by BSE (Palhière, 2011).

Although very little is known about the origin of BSE, it may either occur spontaneously (Eddy, 1995) or if the cattle was fed by scrapie infected sheep, the classical scrapie may cross the species barrier (Wilesmith *et al.,* 1991) and cause deadly mad cow disease (Bovine Spongiform Encephalography, BSE) in cattle (Bruce *et al*., 1997; Hill *et al*., 1997) followed by the spread of the disease from cattle to humans in the form of variant Creutzfeld-Jacob disease (vCJD).

Briefly, if cattle is exposed to scrapie affected tissue it may develop another lethal prion disease (mad cow or BSE), upon consumption by the humans, cattle with BSE may cause another infectious disease in humans in the form of variant Creutzfeldt-Jakob disease (vCJD). Therefore, scrapie has been considered a disease not only affecting the sheep but also causing a serious health problem in other animals and human. Hence, scrapie control has become a prime importance in many countries (Foster *et al.,* 2001).

1. 3. 1. 3 Genetic susceptibility to Classical Scrapie and Programs to Control the Disease

By epidemiological studies, it is concluded that if an animal developed classical scrapie, it had to be not only exposed to an infecting agent, but also genetically susceptible to the disease (Hunter *et al.,* 1997). In other words, the scrapie resistance or susceptibility is largely under genetic control, especially the polymorhisms at codons 136 (valine (V) is associated with high scrapie susceptibility, while alanine (A) is associated with low susceptibility), 154 (arginine (R) is associated with susceptibility, while histidine (H) is associated with partial resistance) and 171 (glutamine (Q) and histidine (H) are associated with susceptibility, while arginine (R) is associated with resistance) of the *PrP* gene are determined to be highly related with the degree of susceptibility or

resistance to classical scrapie in sheep (Laplanche *et al.,* 1993; Hunter *et al.,* 1997; Vaccari *et al.,* 2001; Tongue *et al.,* 2004).

The various combinations of these amino acids produce a variety of alleles and binary combinations of these alleles create various genotypes (Hunter *et al.,* 1996). The individuals having different genotypes show different levels of susceptibility to classical scrapie. Taking advantage of the knowledge of different susceptibility of different genotypes, some breeding programmes were established in many countries (e.g., in Spain (Molina *et al.,* 2006), Portugal (Orge *et al.,* 2003), Greece (Billinis *et al.,* 2004), Italy (Ligios *et al.,* 2006), Slovakia (Holko *et al.,* 2005) and Germany (Luhken *et al.,* 2004) in order to reduce the scrapie risk in national flocks. The aims of these programmes are to protect animal health by reducing and eradicating scrapie -by eliminating sheep with the most susceptible genotype (VRQ/VRQ) to classical scrapie- and by increasing the number of sheep with the more resistant genotypes (Hunter *et al.,* 2007) to improve both animal and public health.

The National Scrapie Plan (NSP) was the first plan which was put in place in the United Kingdom in 2001 (Dawson *et al.,* 1998; Anonymous, 2004; Ibeagha-Awemu *et al.,* 2008) included the Compulsory Ram Genotype Scheme (CRGS) which was designed according to European legislation (Hunter *et al.,* 2007). After the NSP, the European Commission Regulation (EC) No 260/2003 recommended all member states of European Union (EU) to establish genotyping and breeding plans for the eradication of scrapie from affected farms (EC Regulation No 260/2003). According to these plans, the rams should be genotyped, rams with the most susceptible genotypes should be removed, besides, scrapie infected individuals should be culled from the flock and not be used for further breeding.

According to the National Scrapie Plan (NSP), PrP genotypes for classical scrapie were classified in five categories from highly resistant (R1) to highly susceptible (R5) (Hunter

et al., 2007; Dawson *et al.,* 1998; Tongue *et al.,* 2004). The most common 15 genotypes and their degree of resistance/susceptibility are shown in Table 1. 4.

Table 1. 4 Five classes representing different levels of classical scrapie risk. Table adapted from Ulvund (2008).

Risk Level	PrP Genotypes	Degree of resistance/susceptibility
R1	ARR / ARR	Sheep that are genetically most resistant to classical scrapie.
R2	ARR / AHQ ARR / ARH ARR / ARQ	Sheep that are genetically resistant to classical scrapie, but will need carefully selection when used for further breeding.
R3	AHQ / AHQ AHQ / ARH AHQ / ARQ ARH / ARH ARH / ARQ ARQ / ARQ	Sheep that genetically have little resistance to classical scrapie and will need careful selection when used for further breeding.
R4	ARR / VRQ	Sheep that are genetically susceptible to classical scrapie and should not be used for breeding unless in the context of a controlled breeding programme.
R5	ARQ / VRQ AHQ / VRQ ARH / VRQ VRQ / VRQ	Sheep that are highly susceptible to classical scrapie and should not be used for breeding.

Recent studies showed that the ARR allele is detected in scrapie resistant sheep, whereas the VRQ allele exists in scrapie susceptible sheep (Pongolini *et al.,* 2009). Furthermore, sheep with the ARR/ARR genotype are highly resistant to classical scrapie (Ulvund, 2008), while sheep with the VRQ/VRQ genotypes are most susceptible (EC, 2003). Moreover, genotypes carrying the ARQ allele, which is the wild-type allele, are less susceptible to classical scrapie, but they may also develop scrapie (Tranulis, 2002). For this reason, they have intermediate susceptibility. Therefore, in breeding programmes, frequency of the ARR/ARR genotype is tried to be increased and indivudials with the VRQ/VRQ genotype are eliminated.

Some previous studies suggested that the frequency of PrP genotypes may also be associated with the yield characteristics of sheep, in consequence, yield characteristics of sheep breeds may change during the breeding programs (DeVries *et al.,* 2004; Benkel *et al.,* 2007), since the frequency of genotypes are increased or decreased by artificial selection. Though, it was reported that there is no significant evidence of association between PrP genotypes and performance or production traits of sheep (Alexander *et al.,* 2003; DeVries *et al.,* 2004; Vitezica *et al.,* 2005; Brandsma *et al.,* 2004; Man *et al.,* 2006; Isler *et al.,* 2006; Sweeney and Hanrahan, 2008; Moore *et al.,* 2009; Alvarez *et al.,* 2011).

Under these genotyping plans in the countries, a large number of individuals were screened in terms of both *PrP* gene and the presence of classical scrapie. Moreover, sheep breeds were classified in different risk groups according to their genotypes (Drögemüller *et al.,* 2001; Orge *et al*., 2004; Gama *et al.,* 2006; Alvarez *et al.,* 2007; Babar *et al.,* 2008; Karami *et al.,* 2011). These studies revealed a large difference among countries (Ulvund, 2008). For instance, in some countries (e.g., Germany, France, the Netherlands, Lithuania, Hungary) from 20% to 65% of the sheep samples belong to the most resistant group (R1), while in some other countries (e.g., Denmark, Ireland, Norway, Austria, Sweden), the majority of the samples belong to high risk groups (R4 and R5) (EC, 2003; Sviland *et al.,* 2006). In addition to these, it was reported that PrP genotypes belonging to scrapie-resistant groups (R1 and R2) are present at very low frequencies in Pakistani sheep (Babar *et al.*, 2009) and Asian sheep breeds (Ikeda *et al.*, 1995; Lan *et al.,* 2006). Furthermore, the most scrapie-resistant genotype ARR/ARR has been reported at a low frequncy in some breeds of Greek sheep (Billinis *et al.,* 2004), but not in any of Iranian sheep breeds (Frootan *et al.,* 2011). Also, Gombojav et al. (2004) suggested that the genotype ARQ/ARQ associated with susceptibility to classical scrapie belonging to intermediate risk group (R3) was found at very high frequency of 82,6% in Mongolian sheep breeds, while Mongolian sheep was found very poor in scrapie-resistant genotypes.

Although genotypes belonging to R1 and R2 risk groups are known to be resistant to classical scrapie, some classical scrapie cases were found in sheep with these scrapie-resistant genotypes (Ikeda *et al.,* 1995). For instance, 2 natural scrapie cases were detected in sheep with the most classical scrapie-resistant ARR/ARR genotype (Groschup *et al.,* 2007). Moreover, classical scrapie was reported in Suffolk sheep in Europe, with the genotype ARR/ARQ, which is another classical scrapie-resistant genotype (Hunter *et al.,* 1997). It was also reported that nearly 95% of the classical scrapie cases were observed in one of the Spanish sheep breeds with the homozygous genotype ARQ/ARQ, which have an intermediate susceptibility to classical scrapie (Acin *et al.,* 2004).

In some countries (e.g., Germany, France, Spain, Portugal, Greece, Italy, Slovakia), where the guidelines of National Scrapie Plan (NSP) were followed, the controlled breeding policies have been implemented since 2003.

1. 3. 2 Nor98 and Atypical Scrapie

A different type of scrapie than the “classical scrapie” was discovered in Norway in 1998 and defined as "Nor98". In general, this type of scrapie was named as “atypical scrapie” showing different epidemiology, clinical signs, sick animal genotypes and distinct biochemical characters of classical scrapie (Benestad *et al.,* 2003; Konold *et al.,* 2007).

Epidemiologically, Nor98 is observed in 1/10000 of European sheep population. Atypical scrapie occurs in sheep older than 5 years old (OIE *Terrestrial Manual,* 2009), whereas classical scrapie emerges in sheep between the ages 2 and 5. Atypical scrapie is considered to emerge sporadically in older sheep (Lühken *et al.,* 2007).

In atypical scrapie, neurological symptoms are weaker compared to classical scrapie. The accumulation of abnormal prion protein (PrP^{Sc}) is detected in the cerebellum and

cerabral cortex if the animal is infected by atypical scrapie. Moreover, as a result of this accumulation, micro vacuolization is found in the brain in atypical cases instead of actual vacuolization in classical scrapie cases (Benestad *et al.,* 2003). Moreover, the diagnosis of atypical scrapie is more difficult than the classical scrapie. The clinical signs of atypical scrapie were found as tremors, ataxia, changes in temperament and loss of body condition, where ataxia was the first clinical sign detected (Konold *et al.,* 2007).

Some countries (e.g., Germany and France (Buschmann *et al.,* 2004a), Belgium (De Bosschere *et al.,* 2004), Sweden (Gavier-Widen *et al.,* 2004), England (Everest *et al.,* 2006), Switzerland (Nentwig *et al.,* 2007), U.S.A (Loiacono *et al.,* 2009), Portugal (Orge *et al.,* 2004) and Spain (Rodríguez-Martínez *et al.,* 2010)) have began to report atypical scrapie cases and started to focus on this issue. Although the source of atypical scrapie has not been known yet, the studies suggest that polymorphisms both at codons 141 and 154 constitute atypical scrapie susceptibility (Moum *et al.,* 2005; Benestad et al., 2008; Fediaevsky *et al.,* 2009).

1. 3. 2. 1 Genetic susceptibility to Atypical Scrapie

Studies about atypical scrapie suggest that PrP genotypes of atypical scrapie differs from classical scrapie genotypes (Lühken *et al.,* 2007; Benestad *et al.,* 2008). Specifically, the alleles ALHQ (AHQ) and $AF_{141}RQ$ (AFRQ), which is an ARQ variant with Leucine (L) to Phenylalanine (F) mutation at codon 141, have been associated with increased risk of atypical scrapie where the most classical scrapie-resistant allele or genotype is ARR or ARR/ARR, respectively, gives no protection for atypical scrapie (Moum *et al.,* 2005; Arsac *et al.,* 2007; Lühken *et al.,* 2007; Benestad *et al.,* 2008).

With the help of PrP genotyping studies of atypical scrapie, it is reported that genotypes which are relatively resistant to classical scrapie are found to be highly susceptible to atypical scrapie (Benestad *et al.,* 2003). For instance, an atypical scrapie case was found in sheep with the most classical scrapie-resistant genotype (ARR/ARR) (De Bosschere

et al., 2007). These observations raised a concern about the previous selection programs implemented to increase the frequency of classical scrapie-resistant genotypes. Therefore, it is concluded that these eradication programs are needed to be checked and this situation must have also been taken into account before erasing the genotypes.

Countries need to organize a breeding program in order to determine the atypical scrapie risk and the associated genotypes like there is for the classical scrapie. The individuals with different genotypes in atypical scrapie may also represent different risk levels due to variations in susceptibility to atypical scrapie (Fediaevsky *et al.,* 2009).

Table 1. 5 Genotypes of Atypical Scrapie and their risk levels (Fediaevsky *et al.,* 2009).

Risk Level	PrP Genotypes	Group
0	ALRR / ALRQ ALRR / VLRQ ALRQ / ALRQ ALRQ / ALRH ALRQ / VLRQ	**1**
1	ALRR / ALRR ALRR / ALRH VLRQ / VLRQ	**2**
2	ALHQ / ALRH ALHQ / VLRQ AFRQ / ALRH ALRH / ALRH AFRQ / VLRQ ALRH / VLRQ	**3**
3	ALRR / ALHQ ALRR / AFRQ ALHQ / ALRQ AFRQ / ALRQ	**4**
4	ALHQ / ALHQ ALHQ / AFRQ AFRQ / AFRQ	**5**

Similar with the classification of classical scrapie genotypes according to the National Scrapie Plan (NSP), Fediaevsky et al. (2009) classified atypical scrapie genotypes into five risk levels from showing no risk (0) for atypical scrapie to the highest risk (4). The genotypes and atypical scrapie risk levels are given in Table 1. 5 (Fediaevsky *et al.,* 2009).

The genotypes belonging to group 1 are represented by zero (0) level of atypical scrapie, while genotypes that belong to group 4 and group 5 show high levels (3 and 4) of atypical scrapie risk.

There is no available atypical scrapie breeding program which is accepted by all countries yet.

1. 3. 3 Significance of PrP Genotyping Studies for Turkish Sheep Breeds

Since the domestication of sheep, Turkey is subjected to migration of sheep from all directions (Peters *et al.,* 1999). Therefore, Turkish sheep is expected to have high genetic variability and Turkey is the right area to study the PrP genotype variability. Also, PrP genotyping of Turkish sheep have prime importance not only for developing a data at the junction of Asia and Europe for the comparison between Asian and European sheep PrP haplotypes, but also for detecting the unique polymorphisms of the *PrP* gene. In other words, PrP genotyping of Turkish sheep can answer some questions about the evolution of PrP haplotypes.

Some PrP genotyping studies has started in Turkey (Un *et al.,* 2008; Lühken *et al.,* 2008; Elmacı *et al.,* 2009; Alvarez *et al.,* 2011; Frootan *et al.,* 2011; Oner *et al.,* 2011). Firstly, 109 native Turkish sheep belonging to Kıvırcık, Sakız and Gökçeada breeds were genotyped by Un et al. (2008) and it was found that most of the genotypes belong to classical scrapie-resistant risk groups (R1 and R2) and the genotype VRQ/VRQ, which is highly associated with susceptibility to classical scrapie, was absent in the investigated

109 sheep. Kıvırcık and Sakız breeds were reported as having relatively high variability of *PrP* gene with four and five alleles respectively. After that, again a total of 109 individuals that belong to four Turkish sheep breeds (Akkaraman, Dağlıç, Karayaka and Morkaraman) were analyzed by Lühken et al., (2008). It was observed that the ARQ/ARQ, the wild-type genotype, was predominant in all breeds except Akkaraman, while the ARQ/ARH genotype belonging to classical scrapie intermediate risk group (R3) was found to be the most frequent in Akkaraman breed. Additionally, Alvarez et al. (2011) genotyped total of 100 sheep belonging to five Turkish native sheep breeds (Akkaraman, Morkaraman, Tuj, Hemsin and Karayaka). No animals carrying the most susceptible genotype, VRQ/VRQ, was found in sheep examined. Moreover, the wild-type genotype ARQ/ARQ, which belongs to R3 risk group, was the most frequent genotype in these five Turkish sheep breeds analyzed. Similarly, Frootan et al. (2011) found that the ARQ/ARQ genotype had the highest frequency in two native Turkish sheep breeds (Ivesi and Morkaraman) investigated, while the most scrapie-resistant genotype ARR/ARR was absent in these breeds which is an important observation. It was also reported that the VRQ/VRQ, known as the most classical scrapie-susceptible genotype, was observed only in Morkaraman breed at a frequency of 0,027. Lastly, Oner et al. (2011) studied the *PrP* gene polymorhisms of 413 sheep belonging to three native sheep breeds (Kıvırcık, Gökçeada and Sakız) and suggested that the most frequent genotypes were ARR/ARQ and ARQ/ARQ belonging to risk groups R2 and R3. Moreover, the VRQ/VRQ genotype having the highest susceptibility to classical scrapie, was found at very low frequencies in Gökçeada and Sakız in that study.

Even though 10 of Turkish sheep breeds (Akkaraman, Morkaraman, Dağlıç, Karayaka, Sakız, Kıvırcık, Gökçeada, Hemşin, Tuj and İvesi) were genotyped previously, Turkey has more than 15 sheep breeds which are native. Hence, not all breeds have been examined yet. Furthermore, the full Turkish sheep distribution have not been covered, for instance, Güney Karaman, the sheep of Southern Turkey, was not examined before. For this reason, more sheep breeds and individuals covering the whole parts of Turkey

were needed to be studied carefully in order to understand the genetic susceptibility of sheep to disease scrapie in Turkey.

In the present study, prion protein (PrP) gene polymorhisms of 14 native domestic sheep breeds (Norduz, Çineçaparı, Dağlıç, Herik, Kıvırcık, Akkaraman (represented by two independent samples), Sakız, İvesi, Morkaraman, Hemşin, Karagül, Karayaka, Gökçeada and Güney Karaman) at codons 136, 141, 154 and 171 were investigated. Neither classical nor atypical scrapie cases have been reported in Turkish native sheep breeds before. This study including 14 Turkish sheep breeds will considerably fill the gap which is present in the data of geographic distribution of *PrP* gene polymorphisms associated with scrapie, and especially for the atypical one.

1. 3. 4 Goals and Expected Outcomes of the Study

In the present study samples of 14 breeds mostly sampled (579 from 655 individuals) by the personnel of Ministry of Food, Agriculture and Livestock of Turkey were used. Those 579 sheep individuals were sampled for the national project named as; *In Vitro* Conservation and Preliminary Molecular Identification of Some Turkish Domestic Animal Genetic Resources-I (TUBITAK Project No: 106G005) as known with the acronym TURKHAYGEN-I. Moreover, the data for 45 individuals of Güney Karaman breed and data for the previous sample of one of the breeds (Akkaraman) was also obtained and used for comparative purposes. Genotypes of total 655 individuals were determined with respect to polymorphisms at 4 codons of *PrP* gene within the exon 3.

The goals of the present study were:

- To provide a reliable (where breeds are well represented) and extensive (covering many breeds) data on the *PrP* gene polymorphisms in Turkey.

- To understand the distribution of relative risk both associated with classical and atypical scrapie of Turkish native sheep breeds.

It is believed that the knowledge of scrapie risk distribution of sheep in Turkey will be used by the authorities of the Ministry of Food, Agriculture and Livestock, breeders associations and university research units in Turkey to be employed in scrapie control studies or for further research.

This must have been also stated that one of the purposes of TURKHAYGEN-I project was to cryo-conserve tissues (embryo, sperma and somatic cells) of the breeds. They are reserved in the gene banks in order to be used to regenerate the sheep breeds if they will be lost or to support the breeds if they will be too few in number in the future. Since, for Turkish sheep, they will be screened with respect to their resistance/susceptibility status to classical scrapie, the ones to be employed can be selected accordingly.

Again it is believed that, based on the collected data of the present study and the data available in the literature, results will contribute to the understanding of the evolutionary history of ovine *PrP* gene haplotypes (in relation, migration, selection and random genetic drift).

CHAPTER 2

MATERIALS AND METHODS

2. 1 Samples and the Sampling

A total of 655 individuals from 15 sheep populations belonging to 14 Turkish sheep breeds (Norduz, Çine Çaparı, Dağlıç, Herik, Kıvırcık, Akkaraman (represented by two independent samples (Akkaraman-1 and Akkaraman-2)), Sakız, İvesi, Morkaraman, Hemşin, Karagül, Karayaka, Gökçeada and Güney Karaman) were used in this study. Samples of Dağlıç, Kıvırcık, Akkaraman (Akkaraman-1 and Akkaraman-2), Sakız, İvesi, Morkaraman and Güney Karaman were studied in the present study, whereas samples of Norduz, Çine Çaparı, Herik, Hemşin, Karagül, Karayaka and Gökçeada breeds were examined in the context of TUBITAK Project with the Full Name: Surveillance of prion protein gene polymorphisms in Pakistani and Turkish native sheep breeds (TUBITAK –MOST – Project no: 108O708). However, all samples belonging to all breeds were analyzed together in the current study.

Breeds were tried to be collected from different local farms in order to represent the whole gene pool of the breed. Norduz, Çineçaparı, Dağlıç, Herik, Kıvırcık, Akkaraman-1, Sakız, İvesi, Morkaraman, Hemşin, Karagül, Karayaka and Gökçeada were collected by the Ministry of Food, Agriculture and Livestock as a part of a large scale project with the name; *In Vitro* Conservation and Preliminary Molecular Identification of Some Turkish Domestic Animal Genetic Resources-I and with the acronym TURKHAYGEN-I (www.turkhaygen.gov.tr; Project No: 106G115), whereas Güney Karaman was sampled within the present study around Konya and Antalya-Manavgat. Moreover, another sampling which also belong to Akkaraman breed was carried out in the year 2000

around Ankara, Konya and Sivas within the project TUBITAK VHAG1553. Figure 2. 1 represented the distribution of sampling sites for the collected sheep breeds in Turkey.

In Table 2. 1, the names of the breeds and international names in paranthesis, abbreviations of their names, the tail types of the breeds, the project names in which the breeds were sampled and the sample sizes collected for each breed are shown.

Figure 2. 1 Location of the sample sites of the breeds. Figure adapted from Acan, 2012.

Table 2. 1 Description of the samples. Table adapted from Acan, 2012.

Breeds (International Names)	**Abbreviation**	**Tail Type**	**The project name**	**Sample size**
Norduz	NOR	F	TURKHAYGEN-I (TUBITAK-106G115)	42
Çineçaparı	CIC	F		44
Dağlıç	DAG	F		47
Herik	HER	SF		45
Kıvırcık	KIV	T & L		42

Table 2. 1 Continued

Breeds (International Names)	Abbreviation	Tail Type	The project name	Sample size
Akkaraman-1	AKK-1	F		48
Akkaraman-2	AKK-2	F	TUBITAK VHAG1553	31
Sakız (Chios)	SAK	SF	TURKHAYGEN-I (TUBITAK-106G115)	40
İvesi (Awassi)	IVE	F		47
Morkaraman	MOR	F		49
Hemşin	HEM	SF		42
Karagül (Karakul)	KRG	F		43
Karayaka	KRY	T & L		43
Gökçeada (Imbros)	GOK	T & L		43
Güney Karaman	GK	F	The Present Study	45
TOTAL				**655**

F: fat-tail, T &L: thin and long tail, SF: semi-fat tail.

Turkish native sheep breeds can be classified into two main groups according to their tail types: fat tail and thin tail. Fat-tailed breeds are adapted to harsh environmental conditions where the climate change between seasons is higher. These breeds are found in Central, Southern and Eastern Anatolia. On the contrary, thin-tailed breeds are usually encountered on regions where the environmental conditions are warmer. General features of Turkish native sheep breeds which were summarized by General Directorate of Agricultural Research (TAGEM) are given below (TAGEM, 2009).

Norduz: It is a fat tailed breed having some brown or grey colored regions on its white coat. Black spots on head, neck and legs can be seen. It is known that Norduz breed is a

variety of Akkaraman breed. Its primary product is meat, then milk and wool production comes. It is mainly distributed around Van province in Gürpınar county.

Çine Çaparı: It is a fat-tailed breed which is distributed around Aydın province. This breed has been rescued from extinction, which means it experienced a serious bottleneck. Its main use is for milk and then meat production comes.

Dağlıç: Dağlıç is a fat-tailed breed which is used mainly for wool, then meat and milk production. The main distribution of the breed is along Central Western Anatolia. It has a white coat color with occasional black marks around the mouth, nose and eyes.

Herik: It is a thin-tailed breed which is found in the province of Amasya. It also has a white coat color with black marks around mouth, nose, eyes and legs. The primary use for this breed is the meat production, then for the production of milk and wool following. It is a hybrid of Akkaraman, Morkaraman and Karayaka breeds.

Kıvırcık: It is a thin-tailed breed. Its body is generally white. Its main use is for meat or milk, and then wool production comes. Its wool type is classified as carpet wool. It is found in Thrace, Marmara and North Aegean region. Besides, it is thought to be a trans-boundary breed which has been extended in Greece and Bulgaria.

Akkaraman: It is a fat-tailed breed. Its body is white with black speckles at head, nose, ears and legs. Its main use is for meat, then wool and milk production comes. It has a wide range of distribution along Central Anatolia and neighboring regions. This breed has the highest population size in Turkish native breeds.

Sakız: It is a thin tailed breed with some fat at the tail base. It is distributed mainly in the İzmir province of Turkey and Chios Island of Greece. It has some black marks around mouth, nose, eyes and legs on its white body. Its main use is for milk, and then meat production comes.

İvesi: It is a fat-tailed breed. The main use of the breed is milk, and meat following. It is known to be a trans-boundary breed that is also found in Syria. It is mainly distributed in Southern East Anatolia, especially around Şanlıurfa. Moreover, it has low adaptation to humidity and high rate of precipitation.

Morkaraman: Morkaraman is a fat-tailed breed which has a a red or brownish coat color. It is mainly used for meat and distributed in Eastern Anatolia.

Hemşin: Its tail is fat at the base and thin at the tip. It is mainly used for meat and milk production. It is found especially in the provinces of Artvin and Rize in the mountainous regions. Furthermore, it has isolated sub-populations because of the geography of its habitat.

Karagül: It is a fat tailed breed and it has a black coat color. The main distribution of the breed is around Tokat province of Turkey. However, the breed is known to be introduced from Russia first in 1930's and then from Turkmenistan (Erol *et al.,* 2010). Its main use is for meat, then wool and milk productions come.

Karayaka: It is a thin-tailed breed with a white coated body. Its main use is for wool, then meat and milk productions come. It is distributed mainly in the Northern Anatolia, around Tokat and Amasya provinces of Turkey. It is known as its resistance to heavy rain and humidity.

Gökçeada: It is a thin-tailed breed, mainly found at the island of Gökçeada and around Çanakkale province of Turkey. Its body is white with some black spots around eyes, head, ears and legs. Its main use is for milk and then meat productions come.

Güney Karaman: It is a fat-tailed breed. Its main distribution includes Antalya, Mersin, Hatay and Gaziantep. It is well adapted to harsh environmental conditions. Its main use is for milk and meat productions.

Sampling of these breeds were made by taking blood samples from individuals in order to obtain the DNA. ~10 mL of blood were taken into vacuum blood tubes containing K3EDTA to prevent coagulation. After that, blood samples were stored in +4ºC until DNA isolation.

2. 2 Methods

2. 2. 1 DNA Isolation

DNA isolations of samples of sheep breeds except Akkaraman-2 population and Güney Karaman breed were done within the project with the name; *In Vitro* Conservation and Preliminary Molecular Identification of Some Turkish Domestic Animal Genetic Resources-I (TURKHAYGEN-I) (www.turkhaygen.gov.tr; Project No: 106G115), while DNA isolations of the samples belonging to Akkaraman-2 and Güney Karaman were done within the TUBITAK Project with the Full Name: Surveillance of prion protein gene polymorphisms in Pakistani and Turkish native sheep breeds (TUBITAK –MOST – Project no: 108O708). However, all samples belonging to all breeds were analyzed together in the current study.

The DNA samples can be either stored at +4°C (if it is going to be used immediately) or at -20°C (for long term storage in order to prevent the samples from evaporation).

2. 2. 2 Adjustment of DNA Concentrations

The amount and concentration of DNA were checked by agarose gel electrophoresis. 0.8% agarose gels (with 0.5X Tris buffer) were used to check DNA concentrations and their quality.

Firstly, the agarose solution was poured into an electrophoresis plate and waited for approximately ~25 minutes for polymerization. After the polymerization of the gel, the gel was placed into electrophoresis tank which was filled with 0.5X TBE buffer. Secondly, the samples, which were prepared by mixing 3 µL of DNA samples with 3 µL of 6X loading dye (bromophenol blue, sucrose) and 3 µL dH2O, was loaded into the wells of the gel. The gel was run for 40 minutes at 120V. After this, the gels were placed in a EtBr solution for about half an hour. Then, the gels were examined under UV light with transilluminator to decide whether the DNA samples are appropriate to be included in Polymerase Chain Reaction (PCR). The presence, concentrations and the quality of DNA were checked by checking the thickness of bands, presence of smears and the migration patterns of the corresponding bands on the gel. If it was observed that DNA samples had very low amount of DNA, they were not used for the polymerase chain reaction (PCR). New DNA samples were isolated from bloods that belong to the same individuals for PCR. But if the DNA samples had high concentrations, they were diluted. In order to dilute the high concentrated DNA, first of all, samples were incubated for 20 minutes in 55ºC or overnight in 37°C. After the incubation, DNA was taken to a new tube and diluted with Tris-HCl buffer. Diluted samples were checked with 0.8 % agarose gel again. To decide the amount of DNA, which will be used for PCA, sample DNA bands were compared with the bands in the standard size marker (GeneRuler^{TM} DNA Ladder Mix, 100- 1000bp) on agarose gel.

2. 2. 3 Polymerase Chain Reaction (PCR) and Amplification of *PrP* Coding Region

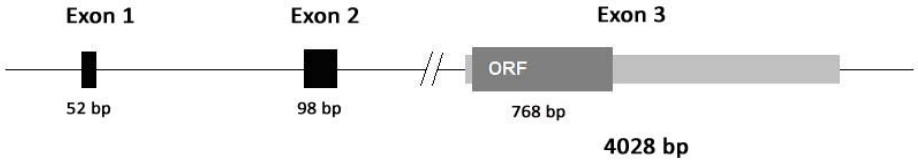
After extracting the DNA, DNA samples were submitted to a polymerase chain reaction (PCR) technique which is based on three steps; denaturation, annealing and extension. The denaturation step includes the separation of DNA's single strands from eachother, whereas primer are bound to their complements on the template DNA in annealing. In the final step, which is extension, primers are extended in the 5'- to – 3' direction.

The 745 bp long *PrP* coding region (Gen-Bank accession number AF195247 from nucleotide 8–752) within the exon three of ovine *PrP* gene which contains three exons (two non-coding and one coding) and two introns with the open reading frame (ORF) located on exon three (Tranulis, 2002; Goldmann, 2008) was amplified. In the present study, for 234 individuals, the *PrP* coding region was amplified by the external primers that was previously used by Babar et al. (2009). On the other hand, the *PrP* coding region of remaining individuals were amplified by the forward and reverse primers that were previously used by Un et al. (2008). Table 2. 2 represented the primers used in this study.

Table 2. 2 The primers used in the present study.

The primer	Primer sequence	Literature
Forward (F)	5'- GAGGAAGAGTTGTGTTACTACT-3'	Babar *et al.,* 2009
Reverse (R)	5'-GTCTGCTTGTCATTTCCCAGTG-3'	
Forward (F)	5'-AAAGCCACATAGGCAGTTG-3'	Un *et al.,* 2008
Reverse (R)	5' -AATGAGGAAAGAGATGAGGAG-3'	

Ovis sp. gene for prion protein PrP, complete cds (GenBank: D38179.1)



4028 bp

Gaattcctttgataaaattcttgttggcatttatatgttcagaggatgttaattccttcatttaacagata
tctattgtttaccttccctgtgccaggctctgccctggcccgctaagaagatagcagcaaacaaagagact
cattccctgctcacattcccacatgaggaaagaggacatgaaccagctattcagaaaattatttcatgatc
tcagcacctaccttggggtcttcccaactggacattagaatcacttccatagggtccatgccagggttcag
aaggttccaggaactaatatcccttataacaacccaataggcagagcttctagggtcctcacaagaacaag
cccagttgcaagaatcactactttaaagaagttcaaagcgtggtaaacctaccagatatttatagtttctt
ccaatttatgatatagtgtaccaatcagaggttatttttatcataagcaattgttgctggcattctgtattt
atcaagttactaggaaatgagccaggaattattttaaggtcaactttgtccttagag (Babar et al.
(2009)) Forward-
GAGGAAGAGTTGTGTTACTACTttacctataattactttatgagatgtatggaatatgagatgtatggaat
gtgaagaacatttatgacctagaatgtttatagctgatgccactgctatacagtcattcattatgctgcag
actttaagtgatttttacgtgggcatttgatgctgacaccctctttattttgcag **EXON 3** agaagtc
atcatggtga Un et al. (2008) Forward-
AAAGCCACATAGGCAGTTGgatcctggttctctttgtggcctatgtggagtgacgtgggcctctgcaagaa
gcgaccaaaacctggcggaggatggaacactgggggagccgatacccgggacagggcagtcctggaggca
accgctatccacctcagggaggggtggctggggtcagccccatggaggtggctggggccaacctcatgga
ggtggctggggtcagccccatggtggtggctggggacagccacatggtggtggaggctggggtcaaggtgg
tagccacagtcagtggaacaagccagtaagccaaaaaccaacatgaagcatgtggcaggagctgctgcagc
tggagcagtggtagggggccttggtggctacatgctgggaagtgccatgagcaggcctcttatacattttg
gcaatgactatgaggaccgttactatcgtgaaaacatgtaccgttaccccaaccaagtgtactacagacca
gtggatcagtatagtaaccagaacaactttgtgcatgactgtgtcaacatcacagtcaagcaacacacagt
caccaccaccaccaaggggagaacttcaccgaaactgacatcaagataatggagcgagtggtggagcaaa
tgtgcatcacccagtaccagagagaatcccaggcttattaccaaaggggggcaagtgtgatcctcttttct
tcccctcctgtgatc Un et al. (2008) Reverse-
CTCCTCATCTTTCCTCATttttctcatagtaggataggggcaaccttcctgttttcattatcttcttaat
ctttgccaggttgggggagggagtgtctacctgcagccctgtagtggtggtgtctcatttcttgcttctct
cttgttacctgtataataataccttggcgcttacag**CACTGGGAAATGACAAGCAGAC-**
Reverse(Babar et al.
(2009))atgagatgctgtttattcaagtcccattagctcagtattctaatgtcccatcttagcagtgatt
ttgtagcaattttctcatttgtttcaagaacacctgactacatttccctttgggaatagcatttctgccaa
gtctggaaggaggccacataatattcattcaaaaaaacaaaactggaaatccttagttcatagacccaggg
tccaccctgttgagagcatgtgtcctgtgtctgcagagaactataaaggatattctgcattttgcaggtta
catttgcaggtaacacagccatctattgcatcaagaatggatattcatgcaacctttgacttatgggcaga
ggacattttcacaaggaatgaacataatacgaaaggcttctgagactaaaaaattccaacatatggaagag
gtgcccttggtggcagccttccattttgtatgtttaagcaccttcaagtgatattcctttctttagtaaca
taaagtatagataattaaggtacc

Figure 2. 2 The binding sites of primers used in this study on ovine *PrP* gene. Figure adapted from the Final TUBITAK Project Report with the Full Name: Surveillance of prion protein gene polymorphisms in Pakistani and Turkish native sheep breeds (TUBITAK –MOST – Project no: 108O708).

Furtherrmore, above figure was prepared in order to visualize the binding sites of the primers used in this study on ovine *PrP* gene. In Figure 2.2, the primers labeled in grey refers to primers previously used by Babar et al. (2009) and primers in turquoise colour shows the primers used by Un et al. (2008).

Table 2. 3 Parameters of the PCR mixture.

With primers previously used by Un et al. (2008)		**With primers previously used by Babar et al. (2009)**	
Parameters	**Added Volume (µl)**	**Parameters**	**Added Volume (µl)**
1X buffer	2,5	1X buffer	2,5
$MgCl_2$	2	$MgCl_2$	2
dNTP	0,5	dNTP	0,5
Primer	0,5	Primer	0,5
Taq Polymerase	0,2	Taq Polymerase	0,2
DNA	3	DNA	2,5
dH_2O	16,8	dH_2O	16,8
Toplam Volume	**25**	**Toplam Volume**	**25**

Table 2. 4 PCR amplification protocol for the PCR with the primers used by Un et al. (2008).

PCR Step	**Temperature (ºC)**	**Duration**	**Number of Cycles**
Denaturation	94°C	2 minutes	1
Denaturation	94°C	1 minute	30
Annealing	57°C	1 minute	
Extension	72°C	1 minute	
Final Extension	72°C	10 minutes	1

Table 2. 3 represented the concentrations of the polymerase chain reaction (PCR) mixture contents both with the primers previously used by Un et al. (2008) and Babar et al. (2009), whereas Table 2. 4 and Table 2. 5 showed the PCR conditions.

Table 2. 5 PCR conditions for the PCR with the primers used by Babar et al. (2009).

PCR Step	Temperature (ºC)	Duration	Number of Cycles
Denaturation	94°C	4 minutes	1
Denaturation	94°C	45 seconds	35
Annealing	54°C	45 seconds	
Extension	72°C	1 minute	
Final Extension	72°C	10 minutes	1

2. 2. 4 Checking the Presence of PCR Products and Purification of PCR products

In order to check the PCR products, 2% agarose gels (with 0.5X Tris buffer) were used. Similar to checking the presence of DNA, the agarose solution was poured into an electrophoresis plate and waited for approximately ~25 minutes for polymerization. Then, the gel was placed into electrophoresis tank filled with 0.5X TBE buffer. For the preparation of the PCR products, 3 µL of DNA samples are mixed with 3 µL of 6X loading dye (bromophenol blue, sucrose), for each PCR product. Samples were loaded into the wells of the gel. The gel was run for nearly 40 minutes at 120V. The gels were placed in a solution that contained EtBr solution and waited for about half an hour. After this, the gels were visualized and photographed under UV light with transilluminator.

After acquiring the PCR products, the PCR products were purified by using PCR Product Purification Kit (Roche, High Pure PCR Product Purification Kit). This principle allows the separation of accompanying substances such as salts, mineral oils, proteins, and other cellular contaminants. So that, the purified DNA can be used directly for sequencing. The procedure used for the purification of PCR products was as follows:

- 500 µl of binding buffer was added on the 100 µl of PCR product. They were mixed well and applied to a pure filter tube. The tube was centrifuged at maximum speed for 1 minute. The flowthrough was discarded.

- 500 µl of washing buffer was added to the tube and centrifuged at 13000xg for 1 minute. The flowthrough was discarded again.
- Then, 200 µl of washing buffer was added to the tube and centrifuged again at 13000xg for 1 minute. The flowthrough and the collection tube were discarded. The filter was transferred to a new sterile tube and 50 µl of elution buffer was added. For the last time, the tube centrifuged at 13000xg for 1 minute. The purified PCR product was achieved.

2. 2. 5 Sequencing

After the purification of PCR products, DNA samples were sequenced with a BigDye^{TM} Cycle Sequencing Kit. After this step, cleaned up DNA samples were analyzed on an automated Applied Biosystems ABI 3100 Genetic Analyzer in which the floorescent signals were translated into nucleotide sequences. The sequencing was carried out in REFGEN Gene Research and Biotechnology Limited in METU-Teknokent.

For the analysis of the sequences from chromatograms, ChromasPro (Technelysium Pty Ltd, http://www.technelysium.com.au/ChromasPro.html) was used. This program allows to open and edit the sequences. Each nucleotide of the sequence was checked by eye in order to acquire the correct sequence of that individual. Figure 2. 3 showed an example of a chromatogram that covers polymorphic codons associated with classical and atypical scrapie.

After editing the sequences with ChromasPro, the sequences were aligned by using BioEdit 5.0.9 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) software according to ClustalW Multiple Alignment method. As well as the equences of the individuals examined in the current study, a reference sequence (GenBank: D38179.1) which was taken from National Center for Biotechnology Information (NCBI) was used.

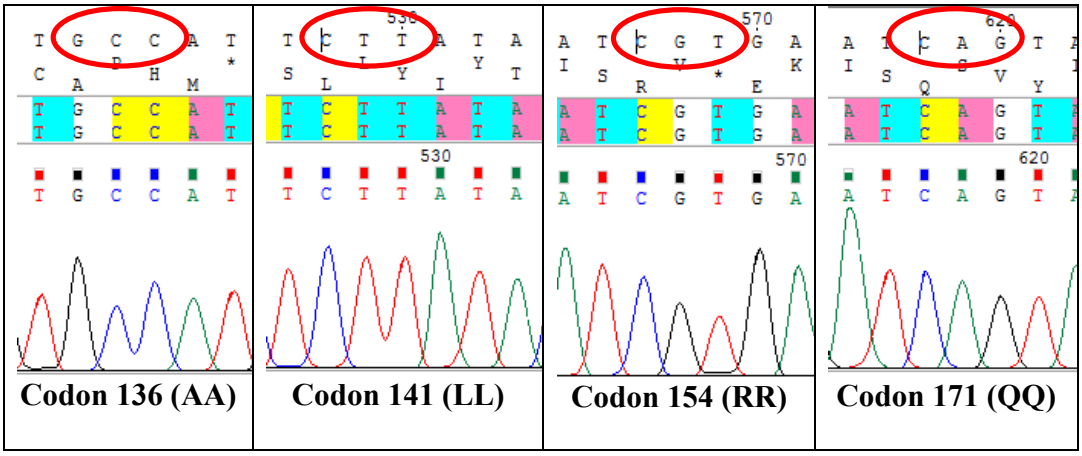


Figure 2. 3 A chromatogram that was used to determine the genotype of the individual Dağlıç 48 (Figure adapted from from the Final Project Report: Surveillance of prion protein gene polymorphisms in Pakistani and Turkish native sheep breeds (TUBITAK – MOST – Project no: 108O708).

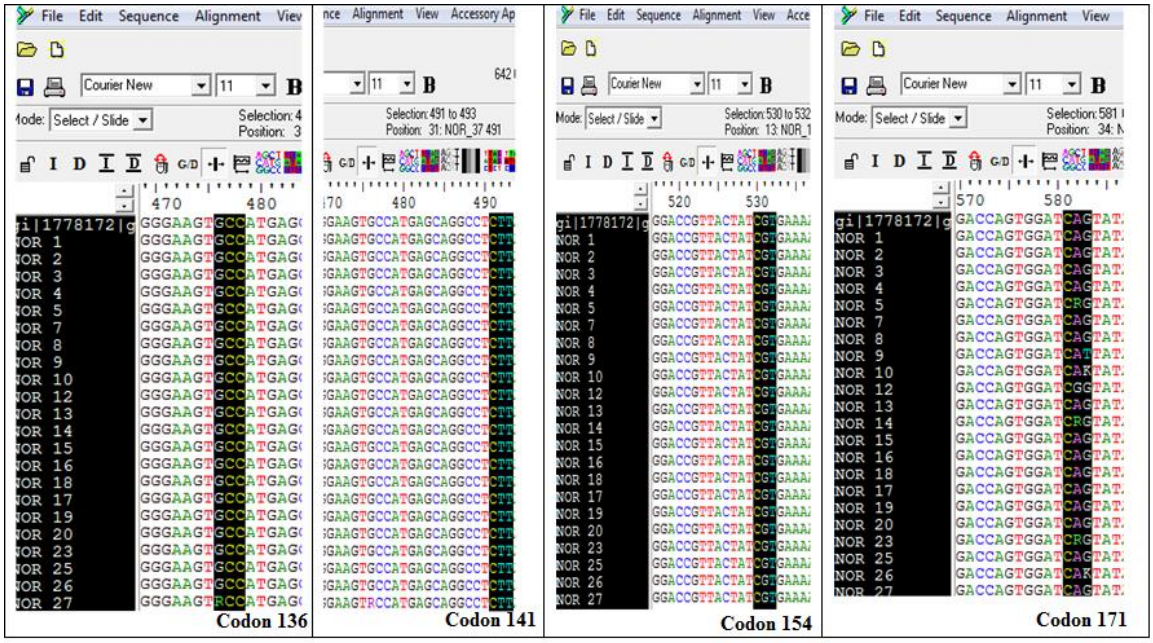


Figure 2. 4 Polymorhisms at codons 136, 141, 154 and 171 of *PrP* gene in BioEdit.

After the alignment of whole sequences, the polymorhisms at codons 136, 141, 154 and 171 were checked directly. Both alleles and genotypes of individuals were recorded. Figure 2. 4 showed the location of polymorphic codons 136, 141, 154 and 171 in BioEdit software.

Furthermore, the polymorhisms, that were present at each of these codons; 136, 141, 154 and 171, were presented in Table 2. 6.

Table 2. 6 Polymorphic nucleotides and amino acids at codons 136, 141, 154 and 171 in Turkish native sheep breeds (Nucleotides differentiated from wild-type are seen in red). Table adapted from the Final TUBITAK Project Report with the Full Name: Surveillance of prion protein gene polymorphisms in Pakistani and Turkish native sheep breeds (TUBITAK –MOST – Project no: 108O708).

Codon	Nucleotide Change	Amino acids
136	**GGA AGT GCC ATG AGC** (wild-type) **ACC** **GTC**	Alanine (A) Threonine (T) Valine (V)
141	**AGG CCT CTT ATA CAT** (wild-type) **TTT**	Leucine (L) Phenylalanine (F)
154	**TAC TAT CGT GAA AAC** (wild-type) **CAT**	Arginine (R) Histidine (H)
171	**GTG GAT CAG TAT AGT** (wild-type) **CAT** **CGG** **AAG**	Glutamine (Q) Histidine (H) Arginine (R) Lysine (K)

2. 3 Statistical Analyses

Statistical analyses were briefly described in this section. The softwares used for these analyses were given in each part after general explanations of the methods.

2. 3. 1 Allele Frequencies

Allele frequency is a measure of the number of copies of a particular allele divided by the number of copies of all alleles at that locus in a population. It describes the amount of genetic diversity at the individual, population, or breed level in the present study.

Allele frequencies of each breed were calculated by using software GenAlex 6.41 (Peakall and Smouse, 2006).

2. 3. 2 Genotype Frequencies

Genotype frequency is the proportion or the frequency of any particular genotype among the individuals of a population. Genotypic frequencies were calculated from the direct counting of genotypes as:

$$f_{ij} = \frac{n_{ij}}{N}$$

where;

n_{ij} = The number of animals with the ij genotype.
f_{ij} = The genotype frequency.
N = The total number of animals in the breed or population (Un *et al.,* 2008).

2. 3. 3 F-statistics: F_{IS} and Pairwise F_{ST} Values

F-statistics, which allow us to measure the genetic differentiation both within and between populations (Allendorf and Luikart, 2007), were developed by Wright (1965) and then extended by Nei (1977). F-statistics consist of three terms which are F_{IS}, F_{IT} and F_{ST} will be explained in detail above. Also, the relationship between these three terms is:

$$1-F_{IT} = (1-F_{IS})x(1-F_{ST})$$

F_{IT} refers to the reduction of heterozygosity due to inbreeding in the total population, whereas F_{IS} is the reduction in heterozygosity again due to inbreeding but in sub-populations. In addition, F_{ST} can be defined as the reduction in heterozygosity due to genetic drift in sub-populations.

Usually in nature, populations do not follow Hardy-Weinberg equilibrium. F-statistics uses these deviations to detect the degree of inbreeding within populations. F_{IS} is the inbreeding coefficient of an individual relative to the sub-population and can be estimated by the below formula:

$$F_{IS} = 1 - \frac{H_O}{H_S}$$

where;

Ho = The mean observed heterozygosity over all sub-populations.
H_S = The mean expected heterozygosity over all sub-populations.

If positive F_{IS} values were estimated, it means that there is inbreeding in the population examined causing heterozygotes deficiency. On the contrary, if F_{IS} values were found to be negative, it implies that there is an excess of heterozygotes because of the migration from outside of the population.

Different from F_{IS}, F_{ST} is the proportion of the total inbreeding in a population due to differentiation among subpopulations and pairwise F_{ST} values can be calculated by the formula:

$$F_{ST} = 1 - \frac{Hs}{H_T}$$

where;

H_S = Mean expected heterozygosity over all subpopulations.
H_T = Expected heterozygosity when all populations are considered as one population.

By using pairs of sub-populations, a distance matrix of pairwise differences between sub-populations can be constituted. Pairwise F_{ST} values will be between 0 and 1, where "0" refers to identical sub-populations and "1" refers to the complete difference between sub-populations, in other words, when F_{ST} is observed as "1", it means that populations are fixed for different alleles. For this reason, F_{ST} is called fixation index.

It is known that, F-statistics, which was proposed by Wright (1965), consider equal finite sample sizes. However, after, Weir and Cockerham (1984) revised the F-statistics in order to resolve it to be suitable for small data sets.

In this study, F_{IS} values within each breed was calculated by FSTAT package program (Goudet, 1995), while F_{ST} values for pairwise comparisons of fourteen breeds were estimated by Arlequin package program (Excoffier *et al.,* 2006). The significance of

these values were tested by applying 000 random permutations and Bonferroni correction by dividing 0.05 by the number of tests performed.

2. 3. 4 Nei's Genetic Distance (D_A) Between The Breeds

Nei's Genetic Distance (DA) is known to be the most appropriate method in order to determine the genetic distances between all sub-populations with respect to each other (Takezaki and Nei, 1996).

It can be calculated by the formula given below:

$$D_A = 1 - \frac{1}{r}\sum_{j}^{r}\sum_{i}^{m_j}\sqrt{x_{ij}y_{ij}}$$

where;

x_{ij} = Frequencies of the i^{th} allele at the j^{th} locus in samples X.
y_{ij} = Frequencies of the i^{th} allele at the j^{th} locus in samples Y.
m_j =Number of alleles at the j^{th} locus.
r =Number of loci examined.

Nei's D_A ranges from 0 to 1. In here, "0" refers to identical sub-populations, whereas "1" refers to sub-populations that share no common alleles. In the present study, Nei's pairwise genetic distances ($D_{A)}$ between fourteen breeds were calculated with the GENDIST program in PHYLIP package software (Felsenstein, 1993).

2. 3. 5 Principal Component Analysis (PCA)

Principal Component Analysis (PCA) is a mathematical procedure that allows to visualize the genetic relationship between populations on independent axises called as principle components. The first principal component explains the highest variation of the data and each succeeding component explains as much of the remaining variation as possible.

In this study, Principal Component Analysis (PCA) was constructed both in 2 and 3 dimensional spaces by Numerical Taxonomy and Multivariate Analysis System (NTSYS) package program (Rohlf, 2000) by using allele frequencies.

2. 3. 6 Neighbor Joining (NJ) Tree

Neighbor joining (NJ) tree is a phylogenetic tree which uses the Neighbor-Joining algorithm of Saitou and Nei (Saitou and Nei, 1987) as the clustering algorithm. NJ Tree is constructed first by forming first two branches by picking two populations which are genetically closest to each other. After that, another population genetically close to first two populations is added as third branch to the tree. All remaining populations is added to the tree by this method.

The different lengths of the branches allow us to understand the relationship between populations. Since neighbor joining (NJ) tree does not assume the same evolutionary rates of the populations, it is considered to be a realistic method for representing the evolution of breeds (Allendorf and Luikart, 2007).

In the present study, the software MEGA (Molecular Evolutionary Genetic Analysis) (Tamura *et al.,* 2008) was used by using both pairwise F_{ST} values and Nei's D_A distances

to construst NJ Trees in order to examine the relationships between 15 sheep populations analyzed.

2. 3. 7 List of Statistical Analysis Methods Applied and the Softwares Used

The list of statistical analysis methods and the softwares used to perform these analyses are given (in paranthesis) below:

- Allele frequencies (GenAlex 6.41)
- F-statistics (FSTAT V.2.9.3, Arlequin 2.001)
- Nei's Genetic Distance (D_A) (GENDIST program in PHYLIP)
- Principle Component Analysis (NTSYSpc)
- Neighbor Joining (NJ) Tree (MEGA)

CHAPTER 3

RESULTS

3. 1 Results of the Laboratory Experiments

3. 1. 1 DNA Extraction and Polymerase Chain Reaction (PCR)

After isolation of the DNA from the blood, samples of the sheep were checked by the agarose gel electrophoresis in order to control the presence, quality and concentration of the isolated DNA. Figure 3.1 represented an example for the agarose gel image of the DNA check which was scanned under UV light. By the gels, concentrations of the DNA samples were observed by comparing sample DNA bands with the bands in the standard size marker (GeneRuler^{TM} DNA Ladder Mix, 100- 1000bp).

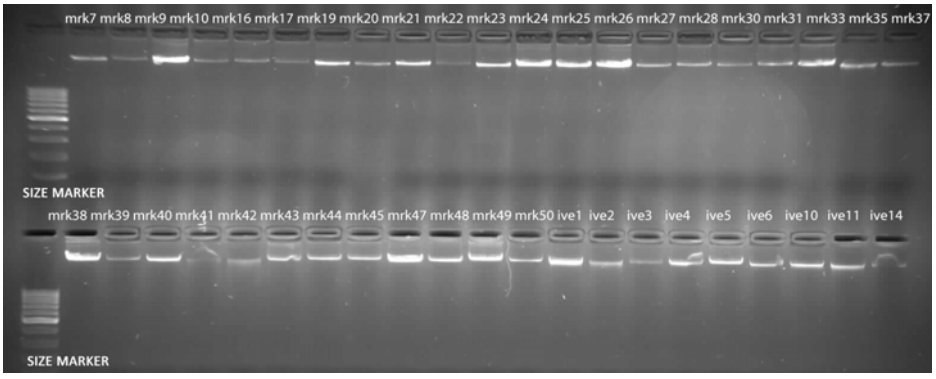


Figure 3. 1 Gel image of total DNA extracts after isolation from the bloods of Morkaraman (mrk) and Ivesi (ive) individuals. Numbers shown above the wells were the DNA sample numbers (SIZE MARKER: GeneRuler^{TM} DNA Ladder Mix, 100- 1000bp).

Incidentally, samples which have very low amount of DNA (samples mrk8, mrk10, mrk16, mrk17, mrk22, mrk41, ive3 and ive14 in Figure 3.1), were not used for the polymerase chain reaction (PCR). New DNA samples were isolated from bloods that belong to the same individuals. In addition, DNA samples (mrk9, mrk24, mrk25, mrk26, mrk38, mrk47 and ive1 in Figure 3.1), which have high concentrations, were diluted as described in materials and methods and checked by agarose gel electrophoresis again. After checking DNA concentrations and adjusting them appropriate for PCR, the extracted DNA was submitted to a polymerase chain reaction (PCR) and *PrP* coding region was amplificated by primers and procedures previously described in materials and methods.

PCR product was visualised after electrophoresis on a 2% agarose gel (Figure 3.2). Figure 3.2 presented an example for the agarose gel image of the amplification results of 745 bp long *PrP* coding region that were scanned under UV light. If samples were not amplified or gave a weak amplification products, they were not used for further analysis, which is sequencing, these samples were amplified again.

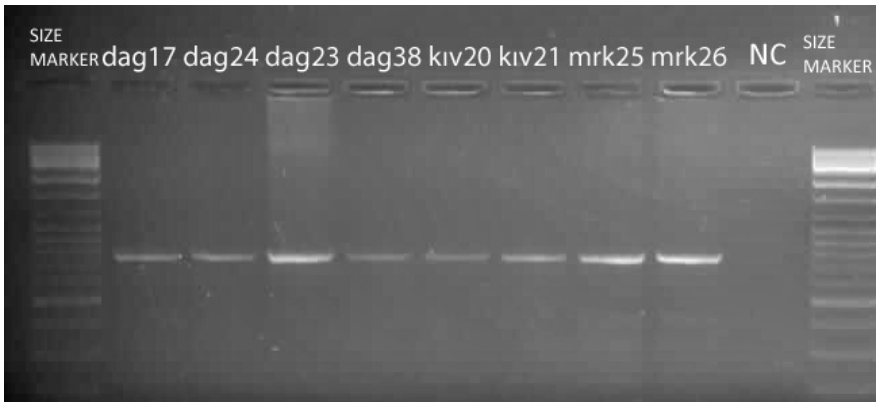


Figure 3. 2 Gel image of PCR amplification products for the *PrP* coding region. Numbers shown above the wells are the DNA sample numbers (dag: Dağlıç breed, kıv: Kıvırcık breed, mrk: Morkaraman breed, NC: Negative Control, SIZE MARKER: GeneRuler^{TM} DNA Ladder Mix, 100- 1000bp).

Moreover, during amplification, a negative control (as also shown in Figure 3.2) was used, which contained PCR mixture without DNA, in order to detect if there was a

contamination in solutions which was used for PCR reactions. Therefore, it was expected to show no bands in agarose gel electrophoresis.

3. 1. 2 Genotyping of *PrP* coding region

Genotyping analysis of *PrP* coding region was carried out by sequencing method which is the determination of the exact order of the nucleotide bases.

Before sequencing, acquired PCR products were purified by using a Roche, High Pure PCR Product Purification Kit. After that, the 745 bp region within exon three of the *PrP* gene was sequenced by an ABI 3100 Genetic Analyzer and a BigDye^{TM} Cycle Sequencing Kit. The sequenced region was edited and aligned as described in materials and methods. Figure 3.3 represented an example for the chromatogram belonging to an individual from Çine Çaparı breed.

Single nucleotide polymorphisms at codons 136, 141, 154 and 171 were checked directly and genotypes were identified.

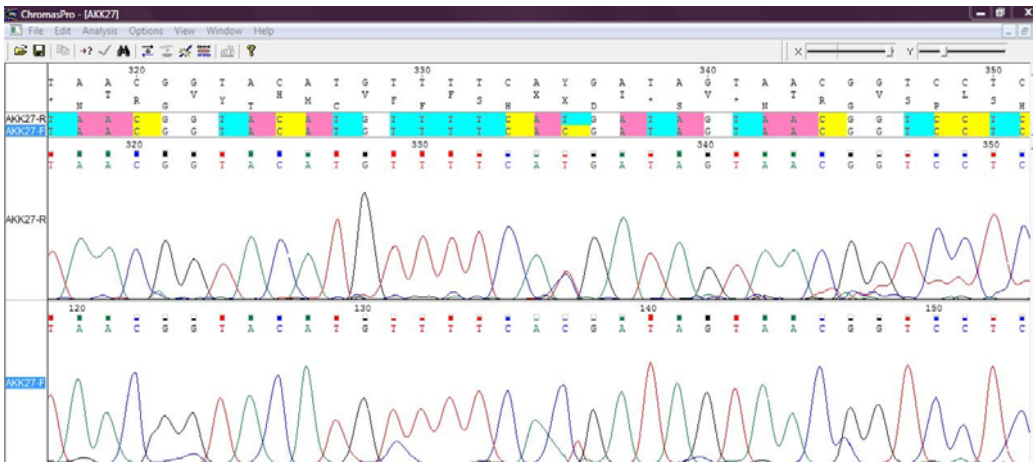


Figure 3. 3 A chromatogram of 27th individual from Akkaraman-1 population.

3. 2 Results of Statistical Analysis

3. 2. 1 Allele Frequencies

Allele frequencies were recorded in two different ways: alleles related with the classical scrapie and alleles for the atypical scrapie.

3. 2. 1. 1 Allele Frequencies of Classical Scrapie

In this study, *PrP* gene polymorphisms of 655 individuals, composed of 15 populations (Norduz, Çineçaparı, Dağlıç, Herik, Kıvırcık, Akkaraman (represented by two independent samples), Sakız, İvesi, Morkaraman, Hemşin, Karagül, Karayaka, Gökçeada and Güney Karaman) were investigated.

Total of 13 different PrP alleles were observed in 655 individuals of Turkish native sheep breeds according to nucleotide changes at codons 136, 154 and 171 which are associated with classical scrapie (Table 3. 1).

Overall in the breeds, the most frequent allele was ARQ (frequency=0.609) in almost all of the breeds, except in Gökçeada, followed by ARR (0.194) and ARH (0.129). The most classical scrapie-resistance allele, ARR, was observed in all of the breeds, but the frequencies were different, ranging from 0.048 in Norduz to 0.510 in Gökçeada. In addition, other two most common alleles, ARQ and ARH, were also observed in all breeds.

Only in Gökçeada, the predominant allele was ARR. In here, it must be remembered that Gökçeada is an island population.

Moreover, the AHQ allele was present in the majority of the breeds (eight out of 14 breeds) studied, at frequencies ranging from 0.011 in Güney Karaman to 0.137 in Sakız.

In the same way, the allele TRQ, which is known to be rare in sheep populations, was observed in 8 of the breeds at a low frequency of 0.01 in Morkaraman and a higher frequency of 0.107 in Karagül.

The VRQ allele, which is associated with the highest susceptibility to classical scrapie, was detected in 5 of the breeds (Çine Çaparı, Dağlıç, Kıvırcık, Karayaka and Gökçeada) with relatively low frequencies. Its frequency ranges from 0.010 in Gökçeada to 0.069 in Karayaka. Overall, it was detected at a low frequency of 0.010.

Table 3. 1 Allele frequencies of classical scrapie in Turkish sheep breeds.

	Sheep Breeds															
PrP Allele	**NOR**	**CIC**	**DAG**	**HER**	**KIV**	**AKK -1**	**AKK -2**	**SAK**	**IVE**	**MRK**	**HEM**	**KRG**	**KRY**	**GOK**	**GK**	**TOTAL**
AHQ	0.024	-	0.068	0.022	0.073	-	-	0.137	-	0.010	-	-	-	0.042	0.011	**0.023**
AHR	-	-	-	-		-	-	-	-	-	-	-	-	0.031	-	**0.002**
ARH	0.158	0.034	0.113	0.022	0.073	0.163	0.403	0.050	0.195	0.204	0.119	0.095	0.093	0.042	0.170	**0.129**
AHH	-	-	-	-		-	-	-	-	0.010	-	-	-	-	-	**0.001**
ARK	-	0.023	0.011	-		0.021	-	0.012	0.032	0.020	-	-	-	-	-	**0.008**
TRH	-	-	-	-		-	-	-	-	-	-	-	0.023	-	-	**0.002**
VRH	-	-	-	-	0.012	-	-	-	-	-	-	-	-	-	-	**0.001**
ARQ	0.756	0.639	0.579	0.700	0.524	0.565	0.500	0.662	0.663	0.602	0.678	0.690	0.546	0.361	0.681	**0.609**
THQ	-	-	-	-		-	-	0.012	-	-	-	-	-	-	-	**0.001**
TRQ	0.012	0.023	-	0.077	-	0.032	-	0.012	0.032	0.010	-	0.107	-	-	-	**0.021**
VRQ	-	0.046	0.011	-	0.048	-	-	-	-	-	-	-	0.069	0.010	-	**0.010**
ARR	0.048	0.232	0.215	0.177	0.268	0.217	0.096	0.112	0.076	0.142	0.202	0.107	0.255	0.510	0.136	**0.194**
VRR	-	-	-	-	-	-	-	-	-	-	-	-	0.011	-	-	**0.001**
n	**42**	**43**	**44**	**45**	**41**	**46**	**26**	**40**	**46**	**49**	**42**	**42**	**43**	**47**	**44**	**640**
PrP Allele Number	**5**	**6**	**6**	**5**	**6**	**5**	**3**	**7**	**5**	**7**	**3**	**4**	**6**	**6**	**4**	**13**

The abbreviations of the breeds are NOR for Norduz, CIC for Çine Çaparı, DAG for Dağlıç, HER for Herik, KIV for Kıvırcık, AKK-1 and AKK-2 for Akkaraman, , SAK for Sakız, IVE for Ivesi, MRK for Morkaraman, HEM for Hemşin, KRG for Karagül, KRY for Karayaka, GOK for Gökçeada and GK for Güney Karaman.

The alleles ARR, AHQ, ARH, ARQ and VRQ, which were mentioned above, are known to be commonly found in sheep populations, so classified as well-known alleles. In the present study, they all were observed in Kıvırcık and Gökçeada breeds. However, the frequency of ARR, the most classical scrapie-resistant allele, was higher in Gökçeada than in Kıvırcık.

Furthermore, some other alleles, which are known to be found rarely in sheep, were also identified in this study. For instance, the allele AHR (0.031) was found only in Gökçeada, whereas the allele VRH (0.012) was present only in Kıvırcık but not in other Turkish native sheep breeds. Another rare allele, VRR, was only found in Karayaka breed at a frequency of 0.011. In addition to these rare alleles, the alleles AHH, TRH and THQ were also found in some of sheep breeds studied. The allele AHH was detected in Morkaraman breed at a frequency of 0.01, while the TRH allele was found only in Karayaka at a frequency of 0.023.

Lastly, the THQ allele, which was found only in Sakız breed (0.012), is a novel allele and never detected in any sheep breeds in any other study so far.

In the light of above observations, it is concluded that the general trends of high ARQ allele frequency and the presence of VRQ allele in the thin-tailed Turkish sheep seem to be robust. Moreover, Sakız and Morkaraman breeds are found to show the highest PrP genetic variability with 7 alleles detected. Akkaraman-2 and Hemşin exhibited the lowest PrP genetic variability harboring only 3 alleles.

3. 2. 1. 2 Allele Frequencies of Atypical Scrapie

According to polymorphisms at codons 136, 141, 154 and 171, fourteen alleles (i.e. AFRQ, ALHQ, ALHR, ALRH, ALHH, ALRK, TLRH, VLRH, ALRQ, TLHQ, TLRQ, VLRQ, ALRR, VLRR) associated with atypical scrapie were recorded, and their frequencies were observed in 14 Turkish native sheep breeds (15 populations). Allele

frequencies of atypical scrapie in Turkish native sheep breeds were presented in Table 3. 2.

Table 3. 2 showed that the most frequent allele was ALRQ (0.609), followed by ALRR (0.194), ALRH (0.125), ALHQ (0.023) and TLRQ (0.021) for atypical scrapie.

The alleles ALHQ and AFRQ, which cause high susceptibility to atypical scrapie, were found in some of the sheep breeds investigated. The ALHQ allele was detected in eight sheep populations out of fifteen, whereas the AFRQ allele was present only in two (Kıvırcık (0.012) and Morkaraman (0.020)) of the populations.

In addition, the alleles TLRQ and ALRK, which their atypical scrapie resistance/susceptibility is not determined yet, was also found in sheep breeds studied. The TLRQ allele (0.021) was found in eight of the populations, whereas the ALRK allele (0.009) was observed in six of the populations at low frequencies. The allele VLRQ, which is the most susceptible allele for classical scrapie, however it causes resistance for atypical scrapie, was detected in five sheep populations (Çine Çaparı, Dağlıç, Kıvırcık, Karayaka and Gökçeada) at relatively low frequencies.

Moreover, some rare alleles associated with atypical scrapie were also found in Turkish native sheep breeds. For example, the ALHH allele (0.010) was present only in Morkaraman, whereas the ALHR allele (0.031) was found only in Gökçeada. Furthermore, the VLRH allele (0.012) was detected only in Kıvırcık, while TLRH and VLRR alleles were observed in Karayaka breed.

Once and for all, one novel allele ($TL_{141}HQ$) associated with atypical scrapie was observed in Sakız breed in this study.

The presence of ALHQ and AFRQ alleles, which are associated with high susceptibility to atypical scrapie, in thin-tailed Turkish sheep seem to be robust.

Table 3. 2 Allele frequencies of atypical scrapie in Turkish sheep breeds.

	Sheep Breeds															
PrP Allele	**NOR**	**CIC**	**DAG**	**HER**	**KIV**	**AKK-1**	**AKK-2**	**SAK**	**IVE**	**MRK**	**HEM**	**KRG**	**KRY**	**GOK**	**GK**	**TOTAL**
AFRQ	-	-	-	-	0.012	-	-	-	-	0.020	-	-	-	-	-	**0.002**
ALHQ	0.024	-	0.068	0.022	0.073	-	-	0.137	-	0.010	-	-	-	0.042	0.011	**0.023**
ALHR	-	-	-	-	-	-	-	-	-	-	-	-	-	0.031	-	**0.002**
ALRH	0.158	0.034	0.113	0.022	0.073	0.163	0.403	0.050	0.195	0.204	0.119	0.095	0.093	0.042	0.170	**0.125**
ALHH	-	-	-	-	-	-	-	-	-	0.010	-	-	-	-	-	**0.001**
ALRK	-	0.023	0.011	-	-	0.021	-	0.0125	0.032	0.020	-	-	-	-	-	**0.009**
TLRH	-	-	-	-	-	-	-	-	-	-	-	-	0.023	-	-	**0.002**
VLRH	-	-	-	-	0.012	-	-	-	-	-	-	-	-	-	-	**0.001**
ALRQ	0.756	0.639	0.579	0.700	0.512	0.565	0.500	0.662	0.663	0.581	0.678	0.690	0.546	0.361	0.681	**0.609**
TLHQ	-	-	-	-	-	-	-	0.012	-	-	-	-	-	-	-	**0.001**
TLRQ	0.012	0.023	-	0.077	-	0.032	-	0.012	0.032	0.010	-	0.107	-	-	-	**0.021**
VLRQ	-	0.046	0.011	-	0.048	-	-	-	-	-	-	-	0.069	0.010	-	**0.010**
ALRR	0.048	0.232	0.215	0.177	0.268	0.217	0.096	0.112	0.076	0.142	0.202	0.107	0.255	0.510	0.136	**0.194**
VLRR	-	-	-	-	-	-	-	-	-	-	-	-	0.011	-	-	**0.001**
n	**42**	**43**	**44**	**45**	**41**	**46**	**26**	**40**	**46**	**49**	**42**	**42**	**43**	**47**	**44**	**640**
PrP Allele Number	**5**	**6**	**6**	**5**	**7**	**5**	**3**	**7**	**5**	**8**	**3**	**4**	**6**	**6**	**4**	**14**

The abbreviations of the breeds are NOR for Norduz, CIC for Çine Çaparı, DAG for Dağlıç, HER for Herik, KIV for Kıvırcık, AKK-1 and AKK-2 for Akkaraman, , SAK for Sakız, IVE for Ivesi, MRK for Morkaraman, HEM for Hemşin, KRG for Karagül, KRY for Karayaka, GOK for Gökçeada and GK for Güney Karaman.

3. 2. 2 Genotype Frequencies

Genotypic frequencies were also recorded related both with the classical scrapie and the atypical scrapie.

3. 2. 2. 1 Genotype Frequencies of Classical Scrapie

In total, 655 animals from 14 sheep breeds (15 populations) were genotyped for the *PrP* gene. PrP genotyping for codons 136, 154 and 171 in 655 sheep revealed a total of 25 genotypes associated with classical scrapie including the wild-type genotype ARQ/ARQ which was detected in all 15 populations.

These genotypes were classified into five different risk groups (R1-R5) (Tongue *et al.,* 2004) according to different levels of susceptibility to classical scrapie as described in the introduction part. Some other genotypes, of which their association with the disease scrapie have not been identified yet, were also observed in the sheep breeds investiged. So, they were classified as "Unidentified Risk Group" (URG). All genotypes were presented in Table 3. 3.

When Table 3. 3 was considered, it was observed that ARR/ARR, the most classical scrapie-resistant genotype, was seen in all of the breeds and it's frequency ranged from 0.023 in Norduz to 0.404 in Gökçeada. Moreover, the other classical scrapie-resistant genotype, ARR/ARQ (0.140) and the wild-type genotype, ARQ/ARQ (0.498) were also detected in all investigated sheep breeds.

Overall, the most frequent genotype was ARQ/ARQ (0.498) belonging to R3 risk group which was previously described in introduction part. The VRQ/VRQ genotype, which is highly associated with susceptibility to classical scrapie, was detected at low frequencies, only in three of breeds (Çine Çaparı (0.022), Kıvırcık (0.023) and Karayaka (0.023)). Furthermore, a heterozygote genotype, ARR/VRQ including the most classical

scrapie-susceptible allele, VRQ and belonging to high-R4 risk group as described in the introduction part, was found in two of the breeds at same frequencies; in Dağlıç (0.021) and in Gökçeada (0.021), whereas another classical scrapie-susceptible genotype, ARQ/VRQ, was observed in three of the breeds (Çine Çaparı (0.045), Kıvırcık (0.047) and Karayaka (0.069).

Table 3. 3 Classical scrapie genotype frequencies in Turkish native sheep breeds and their distribution in the risk groups.

		Sheep Breeds															
Risk Group	**PrP Genotype**	**NOR**	**CIC**	**DAG**	**HER**	**KIV**	**AKK-1**	**AKK-2**	**SAK**	**IVE**	**MRK**	**HEM**	**KRG**	**KRY**	**GOK**	**GK**	**TOTAL**
R1	**ARR/ARR**	0.023	0.113	0.085	0.088	0.190	0.145	0.064	0.025	0.042	0.061	0.119	0.046	0.139	0.404	0.066	**0.117**
R2	**ARR/ARQ**	0.071	0.204	0.191	0.155	0.142	0.104	0.032	0.150	0.063	0.163	0.166	0.093	0.232	0.170	0.133	**0.140**
R2	**ARR/ARH**	-	0.022	-	-	-	0.020	-	-	-	-	-	-	-	-	-	**0.006**
R2	**ARR/AHQ**	-	-	0.021	-	-	-	-	-	-	-	-	-	-	-	-	**0.002**
R3	**ARQ/ARQ**	0.666	0.454	0.404	0.555	0.381	0.479	0.354	0.500	0.574	0.489	0.595	0.581	0.395	0.276	0.600	**0.498**
R3	**AHQ/AHQ**	0.023	-	0.021	-	0.047	-	-	0.075	-	-	-	-	-	0.021	-	**0.011**
R3	**AHQ/ARH**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.022	**0.003**
R3	**ARH/ARH**	0.119	0.022	0.106	-	0.047	0.145	0.290	0.050	0.170	0.183	0.119	0.069	0.069	0.042	0.155	**0.106**
R3	**ARQ/AHQ**	-	-	0.063	0.044	0.047	-	-	0.125	-	0.020	-	-	-	-	-	**0.019**
R3	**ARQ/ARH**	0.071	-	-	0.044	0.023	-	0.096	-	0.042	0.020	-	0.046	-	-	-	**0.023**
R4	**ARR/VRQ**	-	-	0.021	-	-	-	-	-	-	-	-	-	-	0.021	-	**0.002**
R5	**VRQ/VRQ**	-	0.022	-	-	0.023	-	-	-	-	-	-	-	0.023	-	-	**0.003**
R5	**ARQ/VRQ**	-	0.045	-	-	0.047	-	-	-	-	-	-	-	0.069	-	-	**0.011**
URG	**ARR/TRQ**	-	-	-	0.022	-	-	-	0.025	-	-	-	0.023	-	-	-	**0.005**
URG	**ARQ/TRQ**	0.023	0.045	-	0.044	-	0.020	-	-	0.021	0.020	-	0.046	-	-	-	**0.016**
URG	**TRQ/TRQ**	-	-	-	0.044	-	0.020	-	-	0.021	-	-	0.069	-	-	-	**0.011**
URG	**ARR/AHR**	-	-	-	-	-	-	-	-	-	-	-	-	-	0.021	-	**0.002**
URG	**ARH/TRH**	-	-	-	-	-	-	-	-	-	-	-	-	0.046	-	-	**0.003**
URG	**ARH/VRH**	-	-	-	-	0.023	-	-	-	-	-	-	-	-	-	-	**0.002**
URG	**ARK/ARK**	-	-	-	-	-	0.020	-	-	0.021	0.020	-	-	-	-	-	**0.007**
URG	**ARQ/ARK**	-	0.045	0.021	-	-	-	-	0.025	0.021	-	-	-	-	-	-	**0.008**
URG	**ARQ/THQ**	-	-	-	-	-	-	-	0.025	-	-	-	-	-	-	-	**0.002**
URG	**AHQ/AHR**	-	-	-	-	-	-	-	-	-	-	-	-	-	0.042	-	**0.002**
URG	**ARH/AHH**	-	-	-	-	-	-	-	-	-	0.020	-	-	-	-	-	**0.002**

Table 3. 3 Continued

		Sheep Breeds															
Risk Group	**PrP Genotype**	**NOR**	**CIC**	**DAG**	**HER**	**KIV**	**AKK-1**	**AKK-2**	**SAK**	**IVE**	**MRK**	**HEM**	**KRG**	**KRY**	**GOK**	**GK**	**TOTAL**
URG	**VRQ/VRR**	-	-	-	-	-	-	-	-	-	-	-	-	0.023	-	-	**0.002**
UG	**CRK**	-	0.022	0.021	-	0.023	-	0.129	-	0.021	-	-	0.023	-	-	0.022	**0.029**
UG	**MRG**	-	-	0.042	-	-	0.020	0.032	-	-	-	-	-	-	-	-	**0.006**
UG	**MAK**	-	-	-	-	-	0.020	-	-	-	-	-	-	-	-	-	**0.001**
	N	**42**	**44**	**47**	**45**	**42**	**48**	**31**	**40**	**47**	**49**	**42**	**43**	**43**	**47**	**45**	**655**
	Number of Genotypes	**7**	**10**	**11**	**8**	**11**	**10**	**7**	**9**	**10**	**9**	**4**	**9**	**8**	**8**	**6**	**25**

The abbreviations of the breeds are NOR for Norduz, CIC for Çine Çaparı, DAG for Dağlıç, HER for Herik, KIV for Kıvırcık, AKK-1 and AKK-2 for Akkaraman, , SAK for Sakız, IVE for Ivesi, MRK for Morkaraman, HEM for Hemşin, KRG for Karagül, KRY for Karayaka, GOK for Gökçeada and GK for Güney Karaman. Risk groups (R1-R5) shows the increasing level of classical scrapie risk. URG: Unidentifed Risk Group, UG: Unknown Genotype. URG and UG were explained in the text.

Furthermore, the genotypes, ARR/ARH and ARR/AHQ belonging to the R2 risk group, were found at low frequencies in Turkish native sheep breeds. For instance, the ARR/ARH genotype was detected in Çine Çaparı breed (0.022) and Akkaraman-1 population (0.020), whereas ARR/AHQ genotype was found only in Dağlıç breed at a frequency of 0.021.

It was also found that six genotypes belonging to R3 risk group, which is an intermediate classical scrapie risk group, were observed in the studied sheep breeds. One of these genotypes, the AHQ/AHQ was present in five of the populations, while, another one, the AHQ/ARH genotype was found only in Güney Karaman breed. Additionaly, the genotype ARH/ARH (0.106) was detected in all breeds except Herik breed. Genotype ARQ/AHQ was found in five of the populations, whereas the ARQ/ARH genotype was present in seven of the sheep populations.

Among the genotypes of URG, ARH/VRH genotype (0.023) was found only in Kıvırcık, whereas the ARQ/THQ genotype (0.025) was present only in Sakız. In addition, the genotype ARH/AHH (0.020) was detected only in Morkaraman breed. Besides, the genotype ARQ/ARK was found in four of the breeds (Çineçaparı, Dağlıç, Sakız and İvesi) at relatively low frequencies. Moreover, ARR/TRQ (0.005), ARQ/TRQ (0.016) and TRQ/TRQ (0.011), the genotypes that includes TRQ allele, were observed at relatively low frequncies in Turkish sheep. Genotype ARQ/TRQ was found in seven of the populations, whereas TRQ/TRQ was present in four of the populations. In addition, the genotype ARR/TRQ was present in three of the populations; in Herik (0.022), Sakız (0.025) and Karagül (0.023).

Furthermore, the genotypes ARR/AHR and AHQ/AHR were present only in Gökçeada, while the ARH/TRH and VRQ/VRR genotypes were detected only in Karayaka.

The greatest genotype diversity was identified both in Dağlıç and Kıvırcık breeds with eleven genotypes. On the contrary, Hemşin breed, just like it was for the allele diversity,

had the least genotype diversity harbouring only four genotypes associated with classical scrapie.

In addition to these genotype frequency results, in 15 individuals from eight populations (Çine Çaparı, Dağlıç, Kıvırcık, Akkaraman-1, Akkaraman-2, Ivesi, Karagül and Güney Karaman) at codon 171 heterozygosity in two different nucleotides was observed. Therefore, the genotypes of these individuals could not be identified and they were classified as Unknown Genotype (UG) and presented also in Table 3.3.

In terms of nucleotides, M, R and K refers to heterozygote positions of nucleotides. When two of them are present on a codon, amino acid for that codon can not be identified unambigously. Since some individuals exhibited CRK, MRG and MAK combinations at codon 171, not a unique but alternative genotypes can be assigned for those individuals. These possible genotypes were presented in Table 3.4. Of course, detecting a CRK, MRG or MAK at codon 171 causes an amino acid change and this change affects the scrapie-susceptibility and scrapie-resistance of individuals. According to codon 171, the level of risk can be classified as follows; R > Q = H, where R: Arginine, Q: Glutamine and H: Histidine. Figure 3. 4 showed an example of detecting the heterozygosity in two different nucleotides at codon 171 in Dağlıç breed.

As well as determining possible genotypes related with M, K, R, the frequencies of genotypes CRK, MRG and MAK were calculated and shown in Table 3. 5.

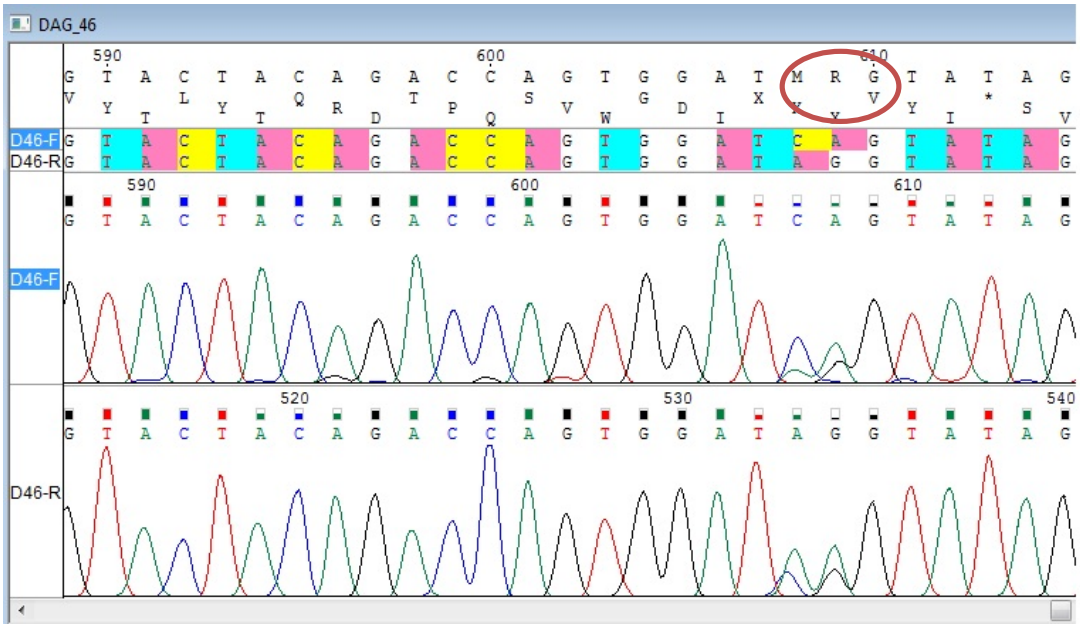


Figure 3. 4 An example of observing a MRG genotype in an individual from Dağlıç breed.

Table 3. 4 Possible genotypes which are the results of detecting heterozygosity in two different nucleotides at codon 171 according to classical and atypical scrapie.

	Codon 136	aa	Codon 141	aa	Codon 154	aa	Codon 171	aa	Genotypes associated with Atypical Scrapie	Genotypes associated with Classical Scrapie
CRK	GCC	A	CTT	L	CGT	R	**CRK** **C(A/G)(G/T)**	**H/R** **Q/R**	ALRR/ALRH ALRR/ALRQ	ARR/ARH ARR/ARQ
MRG	GCC	A	CTT	L	CGT	R	**MRG** **(A/C)(A/G)G**	**K/R** **R/Q**	ALRR/ALRK ALRR/ALRQ	ARR/ARK ARR/ARQ
MAK	GCC	A	CTT	L	CGT	R	**MAK (A/C)A(G/T)**	**Q/K** **H/K**	ALRQ/ALRK ALRH/ALRK	ARQ/ARK ARH/ARK

aa: Amino acid; Amino acids; **A:** Alanine; **L:** Leucine; **R:** Arginine; **H:**Histidine; **Q:** Glutamine; **K:** Lysine

Table 3. 5 Frequencies of CRK, MRG or MAK at codon 171 in Turkish native sheep breeds.

Nucleotide Change	NOR	CIC	DAG	HER	KIV	AKK-1	AKK-2	SAK	IVE	MRK	HEM	KRG	KRY	GOK	GK	TOTAL
CRK	-	0.022	0.021	-	0.023	-	0.129	-	0.021	-	-	0.023	-	-	0.022	**0.029**
MRG	-	-	0.042	-	-	0.020	0.032	-	-	-	-	-	-	-	-	**0.006**
MAK	-	-	-	-	-	0.020	-	-	-	-	-	-	-	-	-	**0.001**
n	**0**	**1**	**3**	**0**	**1**	**2**	**5**	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**1**	**15**

The abbreviations of the breeds are NOR for Norduz, CIC for Çine Çaparı, DAG for Dağlıç, HER for Herik, KIV for Kıvırcık, AKK-1 and AKK-2 for Akkaraman, , SAK for Sakız, IVE for Ivesi, MRK for Morkaraman, HEM for Hemşin, KRG for Karagül, KRY for Karayaka, GOK for Gökçeada and GK for Güney Karaman.

Table 3.5 indicated that the most frequent nucleotide change was CRK at a frequency of 0.029, followed by MRG (0.006) and MAK (0.001). The nucleotide change CRK was present in seven of all the populations, whereas MRG was found in three of the populations. The last nucleotide change, MAK (0.002) was detected only in one population (Akkaraman-1) in two individuals.

3. 2. 2. 2 Genotype Frequencies of Atypical Scrapie

Identification of genotypes associated with atypical scrapie are similar to the identification of genotypes associated with classical scrapie. However, for atypical scrapie, genotypes were identified according to four codons (136, 141, 154 and 171) instead of three codons (136, 154 and 171). All together, 655 animals from 15 populations were genotyped at the PrP locus and polymorhisms at codons 136, 141, 154 and 171 were checked. It was found that Turkish native sheep breeds were having a total of 27 genotypes according to polymorphisms at codons (136, 141, 154 and 171) which are associated with atypical scrapie.

Atypical scrapie genotypes were classified in five risk groups (0-4) according to Fediaevsky et al. (2009) as previously described in the introduction part and presented again in Table 3. 6. Table 3. 6 showed the PrP genotypes of sheep with respect to codons 136, 141, 154 and 171, which are associated with atypical scrapie and their different levels (0 ‑ 4) of atypical risk and their distribution in Turkish native sheep breeds.

In parallel to the classical scrapie results, the most frequent genotype was ALRQ/ALRQ (0.500) belonging to zero (0) risk group, which was described in the introduction part, for atypical scrapie. It was followed by ALRR/ALRQ (0.140) and ALRR/ALRR (0.117). One of the most atypical scrapie-susceptible genotypes, ALHQ/ALHQ was detected in Norduz (0.024), Dağlıç (0.023), Kıvırcık (0.049), Sakız (0.050) and Gökçeada (0.021) breeds. Besides, the other atypical scrapie-susceptible genotype,

AFRQ/AFRQ, was found only in an individual from Morkaraman breed at a frequency of 0.020.

The genotypes, ALRH/ALHH, ALRH/TLRH, VLRQ/VLRR and ALRQ/TLHQ, which their association with atypical scrapie have not been found yet, were classified as Unidentified Risk Group (URG) and also presented in Table 3. 6. Genotype ALRH/ALHH was present only in Morkaraman breed, whereas genotypes ALRH/TLRH and VLRQ/VLRR were found only in Karayaka breed. In addition, the ALRH/VLRH genotype was detected only in Kıvırcık breed, and genotype ALRQ/TLHQ was observed only in Sakız breed.

Table 3. 6 Atypical scrapie genotype frequencies in Turkish native sheep breeds and their distribution in the risk groups.

		Sheep Breeds															
Risk	**Genotype**	**NOR**	**CIC**	**DAG**	**HER**	**KIV**	**AKK-1**	**AKK-2**	**SAK**	**IVE**	**MRK**	**HEM**	**KRG**	**KRY**	**GOK**	**GK**	**TOTAL**
0	**ALRQ/ALRH**	0.071			0.044	0.024		0.115		0.043	0.020		0.047				**0.022**
0	**ALRQ/ALRQ**	0.667	0.465	0.432	0.556	0.366	0.489	0.423	0.550	0.587	0.469	0.595	0.581	0.419	0.277	0.614	**0.500**
0	**ALRQ/VLRQ**		0.047			0.049								0.070			**0.011**
0	**ALRR/ALRQ**	0.071	0.209	0.227	0.156	0.146	0.106	0.038	0.150	0.065	0.143	0.167	0.093	0.209	0.149	0.136	**0.140**
0	**ALRR/VLRQ**														0.021		**0.002**
1	**ALRR/ALRH**		0.023				0.021				0.020				0.021		**0.006**
1	**ALRR/ALRR**	0.024	0.116	0.091	0.089	0.195	0.149	0.077	0.025	0.043	0.061	0.119	0.070	0.163	0.426	0.068	**0.117**
1	**VLRQ/VLRQ**		0.023			0.024											**0.003**
2	**ALHQ/ALRH**															0.023	**0.002**
2	**ALRH/ALRH**	0.119	0.023	0.114		0.049	0.149	0.346	0.050	0.174	0.184	0.119	0.070	0.070	0.043	0.159	**0.106**
3	**ALRQ/ ALHQ**			0.068	0.044	0.049			0.100		0.020						**0.019**
3	**ALRQ/AFRQ**					0.024											**0.002**
3	**ALRR/ALHQ**			0.023													**0.002**
4	**ALHQ/ALHQ**	0.024		0.023		0.049			0.050						0.021		**0.011**
4	**AFRQ/AFRQ**										0.020						**0.002**
URG	**ALHQ/ALHR**														0.043		**0.003**
URG	**ALRH/ALHH**										0.020						**0.002**
URG	**ALRH/ALRK**						0.021										**0.002**
URG	**ALRH/TLRH**													0.047			**0.003**
URG	**ALRH/VLRH**					0.024											**0.002**
URG	**ALRL/ALRL**						0.021			0.022	0.020						**0.005**

Table 3. 6 Continued

		Sheep Breeds															
Risk	**Genotype**	**NOR**	**CIC**	**DAG**	**HER**	**KIV**	**AKK-1**	**AKK-2**	**SAK**	**IVE**	**MRK**	**HEM**	**KRG**	**KRY**	**GOK**	**GK**	**TOTAL**
URG	**ALRQ/ALRK**		0.047	0.023					0.025	0.022							**0.008**
URG	**ALRQ/TLHQ**								0.025								**0.002**
URG	**ALRQ/TLRQ**	0.024	0.047		0.044		0.021			0.022	0.020		0.047				**0.016**
URG	**ALRR/TLRQ**				0.022				0.025				0.023				**0.005**
URG	**TLRQ/TLRQ**				0.044		0.021			0.022			0.070				**0.011**
URG	**VLRQ/VLRR**													0.023			**0.002**
UG	**CRK**	-	0.022	0.021	-	0.023	-	0.1290	-	0.0213	-	-	0.0233	-	-	0.0222	**0.029**
UG	**MRG**	-	-	0.042	-	-	0.0208	0.0323	-	-	-	-	-	-	-	-	**0.006**
UG	**MAK**	-	-	-	-	-	0.0208	-	-	-	-	-	-	-	-	-	**0.001**
	N	**42**	**44**	**47**	**45**	**42**	**48**	**31**	**40**	**46**	**49**	**42**	**43**	**43**	**47**	**45**	**655**
	Number of Genotypes	**7**	**10**	**11**	**8**	**12**	**10**	**7**	**9**	**10**	**10**	**4**	**9**	**8**	**8**	**6**	**27**

The abbreviations of the breeds are NOR for Norduz, CIC for Çine Çaparı, DAG for Dağlıç, HER for Herik, KIV for Kıvırcık, AKK-1 and AKK-2 for Akkaraman, , SAK for Sakız, IVE for Ivesi, MRK for Morkaraman, HEM for Hemşin, KRG for Karagül, KRY for Karayaka, GOK for Gökçeada and GK for Güney Karaman. Risk groups (0-4) shows the increasing level of atypical scrapie risk; URG: Unidentifed Risk Group; UG: Unknown Genotype.

3. 2. 3 Geographical Distribution of Risk Groups

The geographical distribution of risk groups were shown on map of Turkey related with genotypes associated both with classical and atypical scrapie.

3. 2. 3. 1 Geographical Distribution of Classical Scrapie Risk Groups

In order to visualize the distribution of classical scrapie risk in Turkish native sheep breeds, Figure 3. 5 was constituted and each risk group was shown in a different colour. By using genotype frequencies and these colours for different risk groups, pie charts were drawn to visualize the distribution of classical scrapie risk in Turkish sheep breeds (Figure 3. 6).

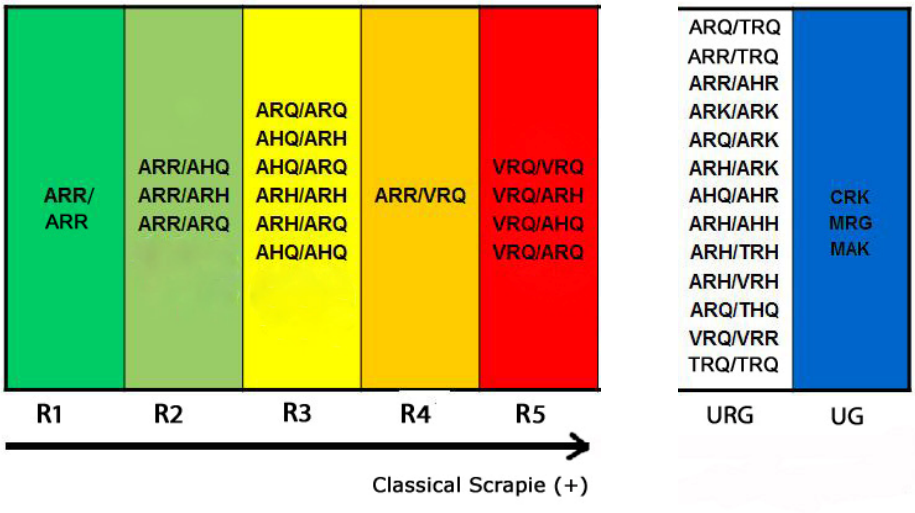


Figure 3. 5 Genotypes associated with classical scrapie and their risk groups according to the classification in National Scrapie Plan (NSP) in Great Britain (Dawson *et al.,* 1998; Tongue *et al.,* 2004; Hunter *et al.,* 2007). Risk Groups (R1-R5) represented the increasing level of classical risk; URG: Unidentified Risk Group; UG: Unknown Genotype.

Overall, results from 655 genotyped individuals revealed that genotypes belonging to R3 risk group (are shown in yellow) are most commonly found in Turkish native sheep breeds. Accompanying genotypes that belong to R1 and R2 risk groups were conferred. Genotype ARR/VRQ, which belongs to high-R4 risk group, was found only in Dağlıç and Gökçeada at a low frequency of 0,021. Furthermore, genotypes in group R5, which is the highest classical scrapie risk group, was observed in Karayaka, Kıvırcık and Çine Çaparı breed. In other words, it was found that Kıvırcık, Dağlıç, Gökçeada, Çine Çaparı and Karayaka breeds have classical scrapie-susceptible genotypes, which means these sheep breeds have higher risk of classical scrapie.

Genotypes, which were previously classified as Unidentified Risk Group (URG), shown in white colour. The last group was shown in blue and named Unknown Genotype (UG) since the possibility of more than one amino acid change at a codon can not be identified as one genotype.

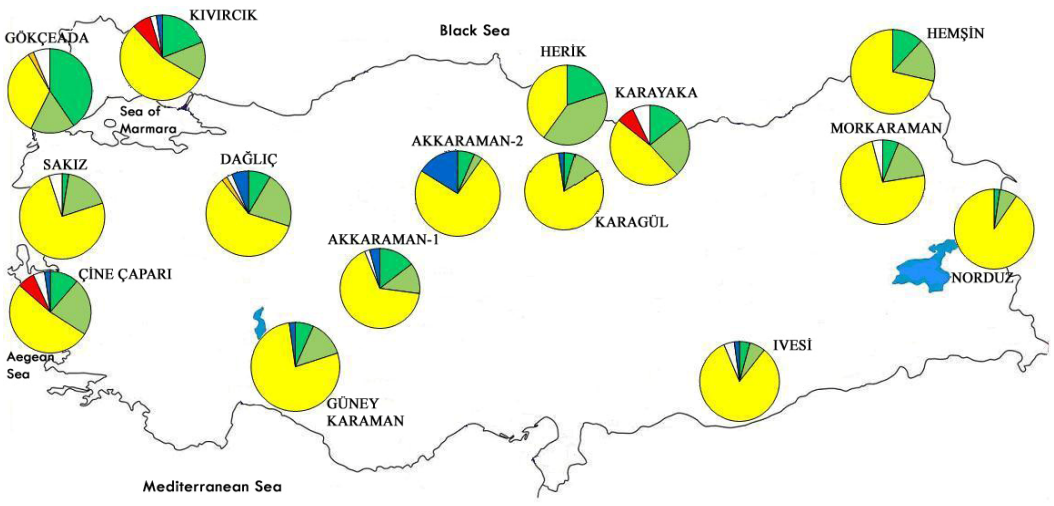


Figure 3. 6 Distribution of risk groups for classical scrapie in Turkish native sheep breeds
(R1: , R2: , R3: , R4: R5: URG: (Unidenfied risk group), UG: (Unknown Genotype))

3. 2. 3. 2 Geographical Distribution of Atypical Scrapie Risk Groups

Such in genotypes associated with classical scrapie, Figure 3. 7 was constituted for atypical scrapie risk and each group was shown in a different range of colour to visualize the distribution of atypical scrapie risk in Turkish native sheep breeds. Moreover, pie charts were drawn to see the distribution of atypical scrapie risk groups in Turkish sheep breeds (Figure 3. 8).

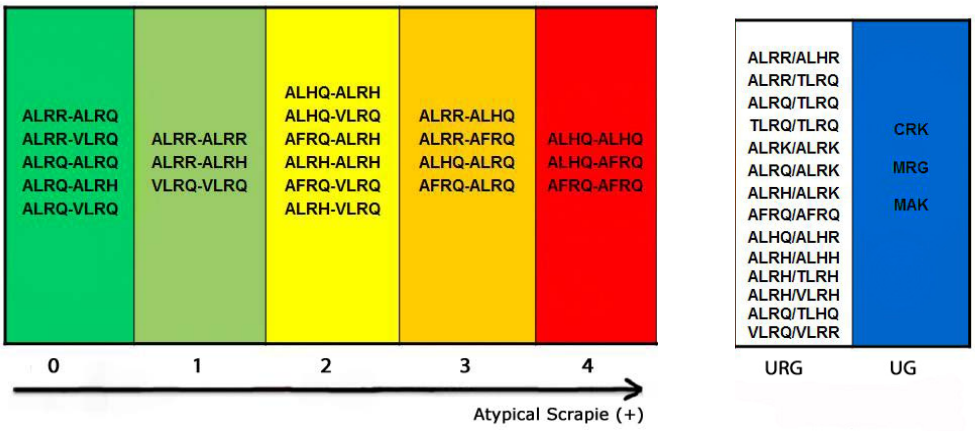


Figure 3. 7 Genotypes associated with atypical scrapie and their risk groups (Fediaevsky *et al.,* 2009). Risk Groups (0-4) represented the increasing level of atypical scrapie risk; URG: Unidentified Risk Group; UG: Unknown Genotype.

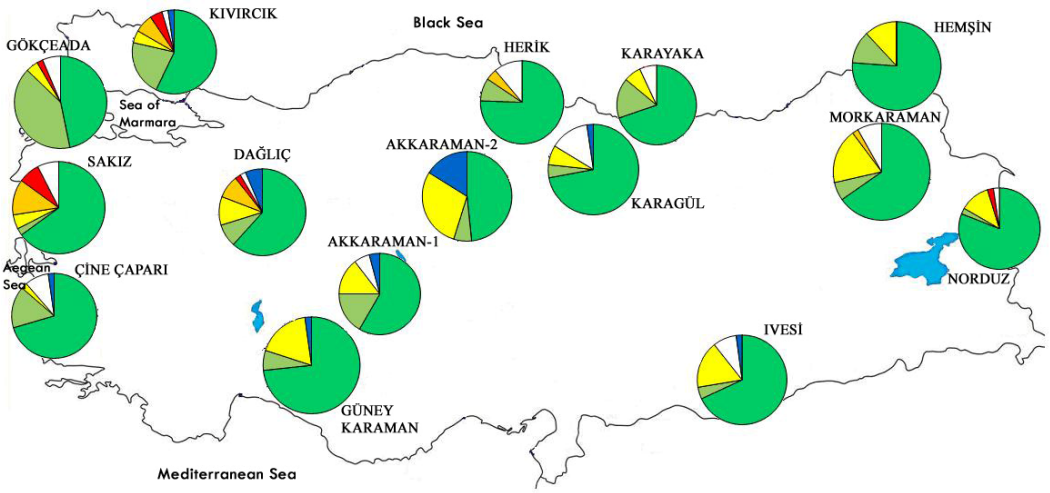


Figure 3. 8 Distribution of risk groups for atypical scrapie in Turkish native sheep breeds. (0 :, 1: , 2:, 3: ,4:, URG: (Unidenfied risk group), UG: (Unknown Genotype))

In relation to atypical scrapie results, the genotypes belonging to zero (0) and one (1) risk groups were frequently seen, followed by genotypes belonging to third (3) risk group in Turkish native sheep breeds. The distribution of genotypes belonging to fourth (4) risk group was detected at very low frequencies in five of Turkish native sheep populations.

In conclusion, high atypical scrapie risk was found in Kıvırcık, Gökçeada, Sakız, Dağlıç, Norduz and Morkaraman breeds.

3. 2. 4 F-Statistics

3. 2. 4. 1 F_{IS} Values

In order to see if the distribution of genotypes associated both with classical and atypical scrapie differed from the Hardy–Weinberg equilibrium, F_{IS} values were calculated by FSTAT package program (Goudet, 1995) and Bonferroni correction was applied.

Results for genotypes associated with classical scrapie and their significance levels were given in the Table 3. 7 below.

Table 3. 7 F_{IS} values and their significance for each breed based on polymorphisms at codons 136, 154 and 171.

Breeds	**N**	**F_{IS} value**
Norduz	42	0.601***
Çineçaparı	44	0.312**
Daglıç	47	0.428***
Herik	45	0.350**
Kıvırcık	42	0.551***
Akkaraman-1	48	0.730***
Akkaraman-2	31	0.743***
Sakız	40	0.347**
Ivesi	47	0.797***
Morkaraman	49	0.558***
Hemsin	42	0.663***
Karagül	43	0.597***
Karayaka	43	0.433***
Gökçeada	47	0.617***
Güney Karaman	45	0.680***

****p<0.01; ***P<0.001**

Similarly, also for genotypes associated with atypical scrapie, F_{IS} values (Table 3.9) were calculated by FSTAT package program (Goudet, 1995) and Bonferroni correction was applied. F_{IS} values were presented in Table 3. 8.

Table 3. 8 F_{IS} values and their significance for each breed based on polymorphisms at codons 136, 141, 154 and 171.

Breeds	N	F_{IS} value
Norduz	42	0.601***
Çineçaparı	44	0.312**
Daglıç	47	0.428***
Herik	45	0.350**
Kıvırcık	42	0.523***
Akkaraman-1	48	0.730***
Akkaraman-2	31	0.743***
Sakız	40	0.340**
Ivesi	47	0.710***
Morkaraman	49	0.569***
Hemsin	42	0.663***
Karagül	43	0.597***
Karayaka	43	0.433***
Gökçeada	47	0.617***
Güney Karaman	45	0.680***

****p<0.01; ***P<0.001**

Considering the F_{IS} values in Table 3. 7 and Table 3. 8, all values were found to be positive and significant ($p < 0.01$, $p < 0.001$). Therefore, it was concluded that the distribution of both classical and atypical genotypes differed from the Hardy–Weinberg equilibrium for all sheep breeds investigated.

3. 2. 4. 2 Pairwise F_{ST} Values

Pairwise F_{ST} values between the populations were estimated according to polymorphisms at codons 136, 141, 154 and 171 by Arlequin package program (Excoffier *et al.,* 2006) and their significance levels were determined. The F_{ST} values are used for a determinant of genetic differentiation between populations, can be seen in Table 3. 9.

According to pairwise F_{ST} values, Gökçeada population was significantly differentiated from other populations in all of the pairwise comparisons. Akkaraman-2 was also relatively distinct from other populations but was not significantly different from Akkaraman-1.

Table 3. 9 Pairwise F_{ST} values of fifteen studied sheep populations based on polymorhisms at codons 136, 141, 154 and 171.

	NOR	CIC	DAG	HER	KIV	AKK-1	AKK-2	SAK	IVE	MRK	HEM	KRG	KRY	GOK	GK
NOR	0.00000														
CIC	0.04085	0.00000													
DAG	0.03426	-0.01084	0.00000												
HER	0.02465	-0.00876	0.00828	0.00000											
KIV	0.07868**	-0.00328	-0.01434	0.02646	0.00000										
AKK-1	0.03369	-0.00850	-0.01641	0.01173	-0.00423	0.00000									
AKK-2	0.07816*	0.08812*	0.04111	0.11992**	0.06221*	0.02725	0.00000								
SAK	0.02465	0.00777	-0.00575	0.00360	0.01158	0.01433	0.08573*	0.00000							
IVE	-0.01208	0.02127	0.00897	0.02208	0.04486*	0.00239	0.02647	0.01936	0.00000						
MRK	0.01372	0.01137	-0.00621	0.02813	0.01684	-0.01363	0.00420	0.01997	-0.01313	0.00000					
HEM	0.01393	-0.01321	-0.00804	-0.00423	0.01328	-0.01201	0.06703*	0.01121	-0.00058	-0.00394	0.00000				
KRG	-0.00816	0.00995	0.01746	-0.01012	0.04919*	0.01474	0.08717*	0.01083	-0.00499	0.01292	0.00002	0.00000			
KRY	0.06343*	-0.01368	-0.01383	0.01800	-0.01632	-0.01284	0.05612*	0.02112	0.02993	0.00652	-0.00144	0.03525	0.00000		
GOK	0.25782***	0.10063**	0.08869**	0.15772***	0.04535*	0.09227**	0.17952***	0.14929***	0.19949***	0.14180**	0.13586**	0.20654***	0.06242*	0.00000	
GK	-0.00766	0.00726	-0.00328	0.01176	0.02760	-0.00642	0.03321	0.00912	-0.01875	-0.01484	-0.01297	-0.00398	0.01491	0.17277***	0.00000

The abbreviations of the breeds are NOR for Norduz, CIC for Çine Çaparı, DAG for Dağlıç, HER for Herik, KIV for Kıvırcık, AKK-1 and AKK-2 for Akkaraman, , SAK for Sakız, IVE for Ivesi, MRK for Morkaraman, HEM for Hemşin, KRG for Karagül, KRY for Karayaka, GOK for Gökçeada and GK for Güney Karaman; *p<0.05; **p<0.01; ***P<0.001.

3. 2. 5 Nei's Genetic Distance (D_A) Between The Breeds

In order to estimate the genetic relationship between populations, Nei's D_A genetic distances for the fifteen populations were calculated with the GENDIST program in PHYLIP package software (Felsenstein, 1993), based on polymorphisms at codons 136, 141, 154 and 171. Nei's D_A genetic distance matrix of the studied populations were presented in Table 3. 10.

The genetic distance ranged from 0.006044 to 0.503870. The lowest genetic distance (0.006044) was between Morkaraman and Güney Karaman, whereas the highest genetic distance (0.503870) was between Gökçeada breed and Akkaraman-2 population.

Table 3. 10 Pairwise Nei's D_A genetic distance values of fifteen studied sheep populations based on polymorhisms at codons 136, 141, 154 and 171.

	NOR	CIC	DAG	HER	KIV	AKK-1	AKK-2	SAK	IVE	MRK	HEM	KRG	KRY	GOK	GK
NOR	0.000000														
CIC	0.056149	0.000000													
DAG	0.045205	0.016347	0.000000												
HER	0.036547	0.011243	0.026252	0.000000											
KIV	0.098847	0.021108	0.012017	0.045955	0.000000										
AKK-1	0.047327	0.025150	0.010849	0.036581	0.029498	0.000000									
AKK-2	0.119287	0.221054	0.142883	0.228261	0.207282	0.100380	0.000000								
SAK	0.028757	0.039696	0.027719	0.023042	0.057057	0.058612	0.210509	0.000000							
IVE	0.006368	0.057280	0.040983	0.044906	0.093453	0.030142	0.080641	0.045153	0.000000						
MRK	0.023362	0.047843	0.022353	0.050345	0.059174	0.010870	0.062120	0.053231	0.009578	0.000000					
HEM	0.026152	0.011867	0.008452	0.016315	0.030798	0.009468	0.137180	0.032477	0.024022	0.015733	0.000000				
KRG	0.016018	0.032129	0.038831	0.011226	0.077184	0.035504	0.162553	0.031447	0.018437	0.032702	0.020950	0.000000			
KRY	0.078313	0.011961	0.014925	0.038815	0.010711	0.017615	0.175169	0.067559	0.069086	0.042642	0.018005	0.058761	0.000000		
GOK	0.465288	0.201035	0.195001	0.280197	0.120706	0.204560	**0.503870**	0.350598	0.424482	0.310340	0.244860	0.375401	0.150654	0.000000	
GK	0.009264	0.032984	0.018451	0.029320	0.056230	0.015899	0.095608	0.032024	0.006552	**0.006044**	0.006972	0.017976	0.039259	0.330138	0.000000

The abbreviations of the breeds are NOR for Norduz, CIC for Çine Çaparı, DAG for Dağlıç, HER for Herik, KIV for Kıvırcık, AKK-1 and AKK-2 for Akkaraman, , SAK for Sakız, IVE for Ivesi, MRK for Morkaraman, HEM for Hemşin, KRG for Karagül, KRY for Karayaka, GOK for Gökçeada and GK for Güney Karaman.

3. 2. 6 Genetic Relatedness of the breeds based on Atypical Allele frequencies

3. 2. 6. 1 Principal Component Analysis (PCA)

In order to visualize above results, Principal Component Analysis (PCA) was constructed both in 2 and 3 dimensional spaces by NTSYS package program (Rohlf, 2000). Atypical scrapie allele frequencies were used in the PCA (Figure 3. 9 and Figure 3. 10).

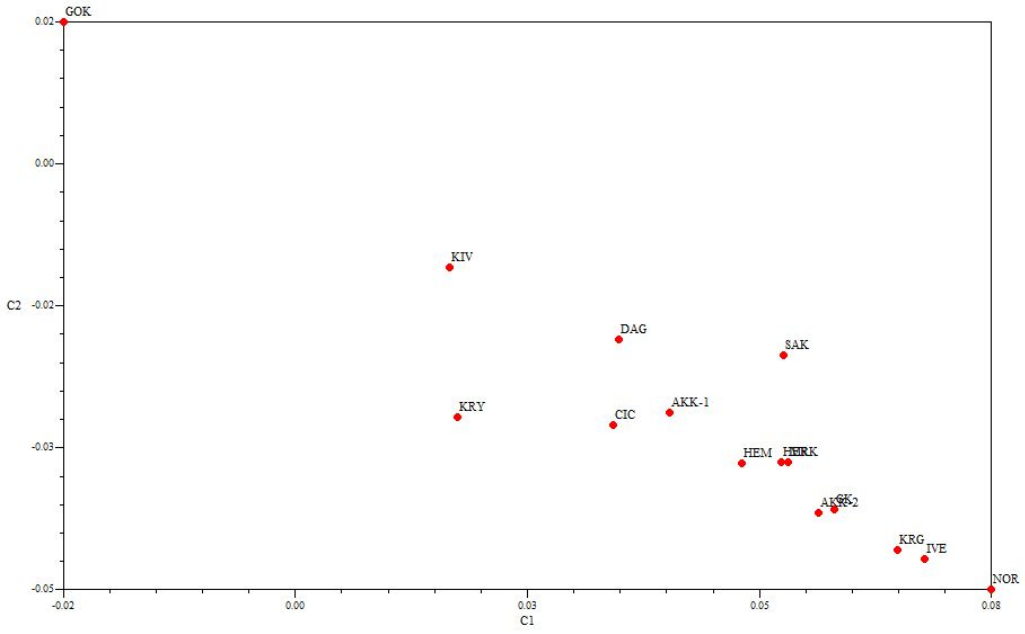


Figure 3. 9 PCA scatter plot of the first and second principal components of 15 sheep populations.

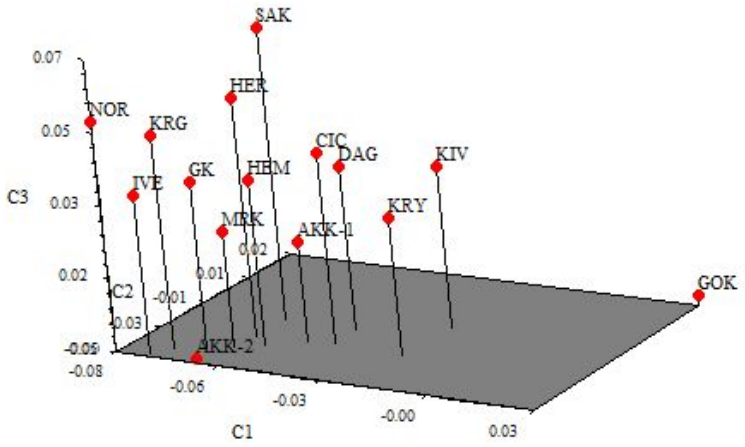


Figure 3. 10 Principal component analysis of 15 Turkish sheep populations in 3 dimentional scale.

In the Principal Component Analysis (PCA), the first three components explained 57,58% of the total variation, with 24,60% explained by the first component, 17,20% by the second component and 15,76% by the third component.

Above PCA result showed the separation of Gökçeada from other populations. Akkaraman-2 population was also separated from other populations especially as seen in Figure 3.10. However, it is relatively close to Akkaraman-1.

3. 2. 6. 2 Neighbor Joining (NJ) Tree of the Breeds

Neighbor joining (NJ) trees were constructed using both pairwise F_{ST} values and Nei's D_A genetic distances in order to determine the relationship between populations.

MEGA package program (Tamura *et al.,* 2008) was used to draw both trees (Figure 3. 11 and Figure 3. 12). Figure 3.11 represents the NJ tree constructed by using the pairwise F_{ST} values, whereas Figure 3. 12 represents the NJ tree constructed by using the Nei's D_A values.

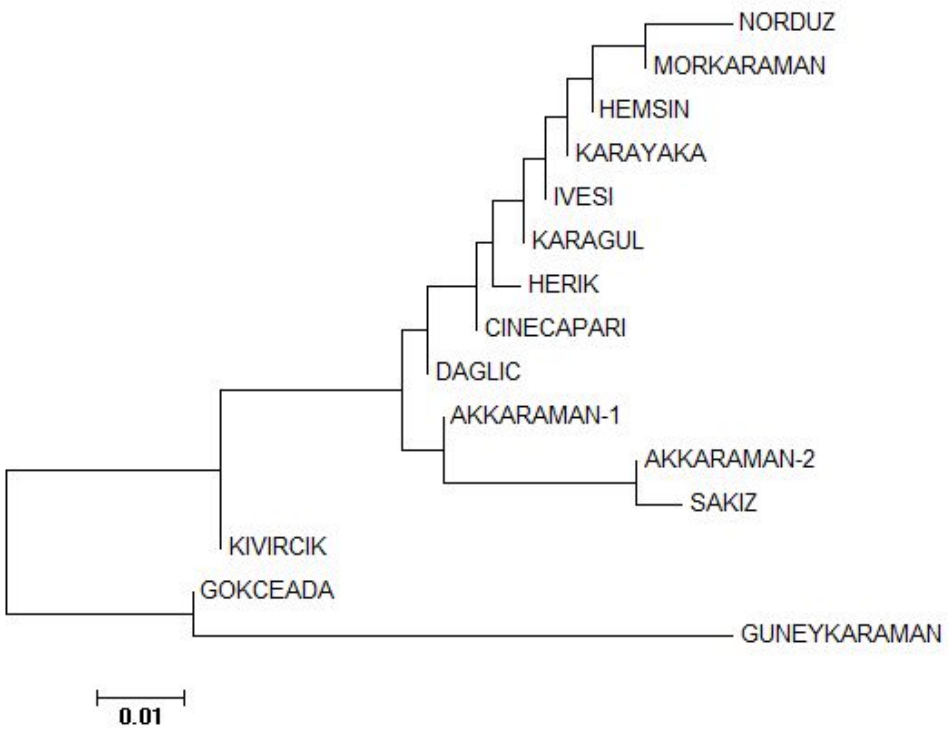


Figure 3.11 Neighbour Joining (NJ) tree that was constructed using the pairwise F_{ST} values between fifteen populations. Atypical scrapie codons were employed.

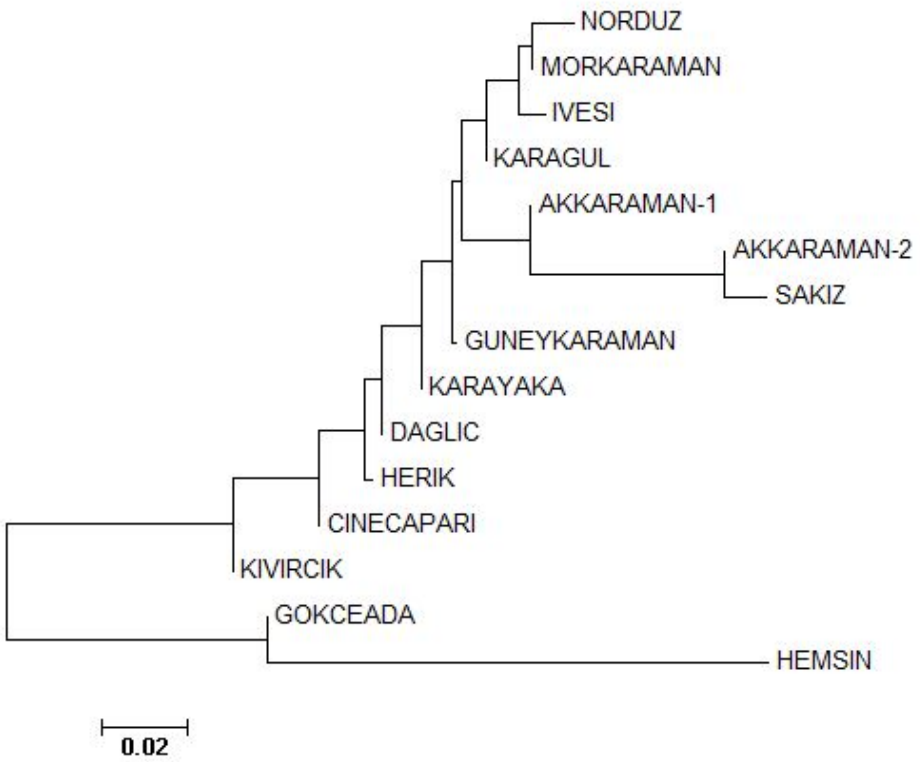


Figure 3.12 Neigbour Joining (NJ) tree that was constructed using the Nei's D_A values between fifteen populations. Atypical scrapie codons were employed.

In these two neighbor joining trees, there were two distinct groups. In the first NJ Tree, which was drawn according to the pairwise F_{ST} values, the first group is composed of thirteen populations except Gökçeada and Güney Karaman, whereas Gökçeada and Hemşin populations were separated in the second NJ Tree which was constructed by using the Nei's D_A genetic distances between the populations.

Akkaraman-1 and Akkaraman 2 populations were together in both of the NJ trees.

Overall, Gökçeada population was observed to be distinct among the examined populations in pairwise comparisons and multidimensional analyses.

3. 3 Scrapie Risk Assessment of Sheep Samples Which are Kept in the Gene Banks

Various tissues of all of the examined samples of the study were already deposited in two different Gene Banks of Turkey in order to be used to regenerate or to support the breeds in the future if it will be needed. One of these gene banks is located in TUBITAK Marmara Research Center in Gebze near Istanbul, whereas the second bank is in Ankara at Livestock Research Institute of Lalahan.

All the individuals were screened with respect to their resistance/susceptibility status to classical scrapie and in accordance with the European Union Commission Regulation with the number 727/2007, in order to have breeds with minimum classical scrapie risk, males without scrapie risk and females not to be employed in the development of the next generation were identified and their gene bank codes were presented in Table 3. 11 and Table 3. 12, respectively.

Table 3. 11 Males and their codes, to be used in order to reduce the risk of classical scrapie.

Males with the ARR/ARR genotype	
Sample name	**Code of the sample**
Norduz	
NOR 12	Ovis-Nor-01-03-2009-65-275E-01
Çine Çaparı	
CIC 14	Ovis-Çiç-01-03-2008-09-961E-03
CIC 17	Ovis-Çiç-01-03-2008-09-968E-03
CIC 21	Ovis-Çiç-01-03-2008-09-976E-03
Dağlıç	
DAG 44	Ovis-Dağ-01-03-2007-03-44E-10
DAG 49	Ovis-Dağ-01-03-2007-03-49E-03
Herik	
HER 33	Ovis-Her-01-03-2009-05-33E-05

Table 3. 11 Continued

Males with the ARR/ARR genotype	
Sample name	**Code of the sample**
Kıvırcık	
KIV2	Ovis-Kıv-03-03-2008-39-02E-03
KIV7	Ovis-Kıv-03-03-2008-39-07E-05
KIV13	Ovis-Kıv-03-03-2008-39-13E-05
KIV16	Ovis-Kıv-03-03-2008-39-16E-09
KIV17	Ovis-Kıv-03-03-2008-39-17E-09
KIV21	Ovis-Kıv-03-03-2008-39-21E-03
Akkaraman	
AKK 34	Ovis-Akk-01-03-2007-42-34E-09
AKK 37	Ovis-Akk-01-03-2007-42-37E-12
AKK 41	Ovis-Akk-01-03-2007-42-41E-16
AKK 49	Ovis-Akk-01-03-2007-42-49E-24
Karagül	
KRG 43	Ovis-Krg-01-03-2009-60 43E-02
Morkaraman	
MRK 14	Ovis-Mrk-04-03-2008-25-265E-05
Hemşin	
HEM 26	Ovis-Hem-01-03-2008-08-TR084359E-16
HEM 31	Ovis-Hem-01-03-2008-08-TR085737E-03
Karayaka	
KRY 34	Ovis-Kry-01-03-2007-60-34E-09
KRY 44	Ovis-Kry-01-03-2007-52-44E-19
Gökçeada	
GOK 2	Ovis-Gökça-04-03-2007-17-02E-02
GOK 7	Ovis-Gökça-04-03-2007-17-07E-02
GOK 13	Ovis-Gökça-04-03-2007-17-13E-04
GOK 14	Ovis-Gökça-04-03-2007-17-14E-04
GOK 17	Ovis-Gökça-04-03-2007-17-17E-04
GOK 19	Ovis-Gökça-04-03-2007-17-19E-04
GOK 21	Ovis-Gökça-04-03-2007-17-21E-04
GOK 22	Ovis-Gökça-04-03-2007-17-22E-04
GOK 23	Ovis-Gökça-04-03-2007-17-23E-04
GOK 24	Ovis-Gökça-04-03-2007-17-24E-04
Güney Karaman	
GK 7	Ovis-GK-05-03-2011-TR42-9893E-01
GK 17	Ovis-GK-05-03-2011-TR42-9950E-01
GK 31	Ovis-GK-05-03-2011-TR42-8734E-01

Table 3. 12 Females and their codes, not to be used in order to reduce the risk of classical scrapie.

Females carrying VRQ allele (with the genotypes: ARQ/VRQ, ARR/VRQ, VRR/VRQ, VRQ/VRQ)	
Sample name	**Code of the sample**
Çine Çaparı	
CIC 2	Ovis-Çiç-01-03-2008-09-585-01
CIC 28	Ovis-Çiç-01-03-2008-09-536-01
Kıvırcık	
KIV 38	Ovis-Kıv-03-03-2008-39-38-06
KIV 44	Ovis-Kıv-03-03-2008-39-44-08
Karayaka	
KRY 6	Ovis-Kry-01-03-2007-60-06-42
KRY 14	Ovis-Kry-01-03-2007-60-14-35
KRY 18	Ovis-Kry-01-03-2007-60-18-39
KRY 23	Ovis-Kry-01-03-2007-60-23-16
Dağlıç	
DAG 15	Ovis-Dağ-01-03-2007-03-38-15

CHAPTER 4

DISCUSSION

In native Turkish sheep breeds, both classical and atypical scrapie have never been diagnosed before. The reasons are most probably; the long incubation period of the disease (Dickinson *et al.,* 1968) and lack of suitable screening systems for diagnosis (Babar *et al.,* 2009). Moreover, different environmental factors (Stevens *et al.,* 2009; Imrie *et al.,* 2009) and certain flock characteristics may also be associated with the disease scrapie (Healy *et al.,* 2004). Above all, it was understood that there is an association between *PrP* gene polymorphisms and the risk of classical/atypical scrapie in sheep (Laplanche *et al.,* 1993; Hunter *et al.,* 1997). So, the most significant reason for not detecting neither a classical nor an atypical scrapie case in Turkey might be Turkish native sheep breeds are having lower susceptibility to classical scrapie compared to European sheep.

The research described in the present study was carried out to determine the *PrP* gene haplotype distribution in Turkey both associated with classical and atypical scrapie in order to understand the classical and atypical scrapie risk level in Turkish native sheep.

In 2001, the National Scrapie Plan (NSP), which is a long-term programme to control or eradicate classical scrapie from national sheep flocks, was put in place in Great Britain. According to this plan, the rams had to be genotyped, hence, rams with the most classical scrapie-susceptible genotypes had to be removed from flocks so that they will not contribute to the next generation. The main goals of the NSP are to eliminate the most susceptible allele to classical scrapie, VRQ, and

increase the number of sheep with the most resistant genotype, ARR/ARR (Hunter *et al.,* 2007).

Many European countries, such as Germany (Lühken et al., 2004), Greece (Billinis *et al.,* 2004), Italy (Ligios *et al.,* 2006), the Netherlands (Nodeljik *et al.,* 2011), France (Elsen *et al.,* 1999), Spain (Molina *et al.,* 2006), Portugal (Orge *et al.,* 2004), Slovakia (Holko *et al.,* 2005) and Iceland (Thorgeirsdottir *et al.,* 1999), have also developed their own scrapie eradication programmes since 2003 by taking into account the guideline of National Scrapie Plan (NSP). All these breeding programmes provided opportunity to screen over 2 million sheep until today (Hunter *et al.,* 2007).

Some recent studies provided direct evidence for the efficiency of these breeding programs (Leymarie *et al.,* 2009; Melchior *et al.,* 2010; Nodeljik *et al.,* 2011). For instance, in Nodeljik et al. (2011), 1175 sheep were monitored in the Netherlands and it was observed that the frequency of both the most classical scrapie-resistant allele and the genotype, ARR and ARR/ARR, respectively, increased and observed classical scrapie cases decreased in flocks identified in the study. Furthermore, French sheep were genotyped in the course of a breeding program which resulted in observing many scrapie-resistant and no VRQ-carrier rams (Leymarie *et al.,* 2009). In addition to these, Wisniewska and Mroczkowski (2009) was another study in which higher frequencies of ARR/ARR genotypes were observed in Polish sheep according to some simulations to understand the expected frequencies of this genotype in next generations.

The classical scrapie-resistant genotypes are found to be susceptible to atypical scrapie (Buschmann *et al.,* 2004b; Moum *et al.,* 2005). Hence, while creating scrapie-resistant flocks by previous breeding programmes, risk of generating flocks which are sensitive to atypical scrapie became of a concern. Another fear was, in relation to possible reduction in some production traits, during the

directional selection towards the scrapie resistant flocks. However, no negative association was found between the occurrence of PrP resistant alleles and genotypes and production traits of sheep (DeVries *et al.,* 2004; Brandsma *et al.,* 2005; Sweeney and Hanrahan, 2008; Sawalha *et al.,* 2009; Alvarez *et al.,* 2011).

Understanding the existence of atypical forms of scrapie (for instance: Nor98) in classical scrapie-resistant sheep, in other words, the sheep selected from breeding programs for classical scrapie control may have higher susceptibility to atypical scrapie, revealed the need for a comprehensive study of association between the atypical scrapie as well as classical scrapie and *PrP* gene polymorphisms.

Although, no breeding program for scrapie control has been established in Turkey, PrP genotyping studies have started in 2008 (Un *et al.,* 2008) and continued with some other studies (Lühken *et al.,* 2008; Alvarez *et al.,* 2011; Frootan *et al.,* 2011; Oner *et al.,* 2011). Between 2008 and 2011, total 800 individuals from ten of Turkish sheep breeds (Akkaraman, Morkaraman, Dağlıç, Karayaka, Sakız, Kıvırcık, Gökçeada, Hemşin, Tuj ve İvesi) were genotyped (Un *et al.,* 2008; Lühken *et al.,* 2008; Alvarez *et al.,* 2011; Frootan *et al.,* 2011; Oner *et al.,* 2011).

In the present study, the complete coding region of the *PrP* gene in 14 Turkish native sheep breeds (Norduz, Çineçaparı, Dağlıç, Herik, Kıvırcık, Akkaraman (represented by two independent samples), Sakız, İvesi, Morkaraman, Hemşin, Karagül, Karayaka, Gökçeada and Güney Karaman) were analyzed and the genotypes associated both with classical and atypical scrapie were determined. Moreover, these genotypes were classified into risk groups. By this way, distributions of both classical and atypical scrapie risk were determined in Turkey.

4. 1 Distribution of Allele Frequencies

Results of the present study were compared with those of the previous studies from both in Turkey and around the world in order to interpret the distribution of allele frequencies of both classical and atypical scrapie in Turkey.

When the polymorhisms at codons 136, 154 and 171, which are known to be associated with classical scrapie, were considered, in the study, the most frequent allele was ARQ (0,609), which is the wild-type allele. This was a similar observation with what was observed from all of the previous studies carried out on Turkish native sheep breeds (Un *et al.,* 2008; Lühken *et al.,* 2008; Alvarez *et al.,* 2011; Frootan *et al.,* 2011; Oner *et al.,* 2011). These results support the hypothesis that ARQ allele is the wild-type allele in sheep and all remaining alleles occurred as a result of a mutation in the ancestral ARQ allele (Tranulis, 2002).

As it was also mentioned in the results part, the ARQ allele, which is also one of the susceptible alleles for classical scrapie, was predominant in all of the studied sheep breeds except Gökçeada in the present study. The most classical scrapie-resistant, ARR allele was predominant and ARQ was the second most common allele in Gökçeada breed. This slightly different observation occurs probably because Gökçeada is an island population, which makes it an isolated breed, so it might be strongly affected by random genetic drift. For these reasons, detecting the ARQ allele at a different frequency in Gökçeada compared to other breeds is not surprising.

The ARQ allele was the most common allele in Asian (Lan *et al.,* 2006; Wang *et al.,* 2008; Babar *et al.,* 2009; Tsunoda *et al.,* 2010; Karami *et al.,* 2011) and most of the European sheep (Drögemüller *et al.,* 2001; Sipos *et al.,* 2002; Acutis *et al.,* 2004; Gama *et al.,* 2006; Sawalha *et al.,* 2007; Gorjanc *et al.,* 2010; Sirakov *et al.,* 2011), also. This might be also expected, since it is assumed that domesticated

sheep migrated from Middle East to Asia and Europe (Zeder, 2008), during this migration, sheep carrying the wild-type allele, ARQ, must be the founders of native sheep of Asia and Europe.

The second most frequent allele was ARR (0,194), which is the most classical scrapie-resistant allele, and it was present in all sheep breeds investigated in this study. This allele was also detected as the second most common allele in Un et al. (2008), Alvarez et al. (2011) and Oner et al. (2011), however, in Morkaraman and Ivesi breeds investigated in Frootan et al. (2011), it was reported that the allele ARH, which is also associated with classical scrapie resistance, was the second frequent allele, followed by ARR. Although the frequency of ARR varies in sheep investigated, it was observed in all previous studies of Turkish native sheep breeds. However, only having the ARR allele at relatively high frequencies is not enough to be classified as scrapie-resistant.

The VRQ allele, which is associated with the highest susceptibility to classical scrapie, was present in 5 of the studied breeds (Çine Çaparı, Dağlıç, Kıvırcık, Karayaka and Gökçeada) with relatively low frequencies as previously mentioned in the results part. Its frequency ranges from 0,010 in Gökçeada to 0,069 in Karayaka. Overall, it was detected at a low frequency of 0,010. In all previous studies of Turkish native sheep, similar frequency values (0,004 - 0,017) for this allele was reported (Un *et al.,* 2008; Lühken *et al.,* 2008; Alvarez *et al.,* 2011; Frootan *et al.,* 2011; Oner *et al.,* 2011). These results may indicate the detection of most classical-scrapie susceptible allele, VRQ, at very low frequencies in Turkish sheep. However, the frequency of VRQ is much higher in European sheep (François *et al.,* 2003; Acutis *et al.,* 2004; Alvarez *et al.,* 2009; Hagenaars *et al.,* 2010; Sirakov *et al.,* 2011) with a range of 0,002 in Germany (Lühken *et al.,* 2004) and 0,157 in Romania (Otelea *et al.,* 2011)). The breeds exhibiting VRQ allele in Turkey are either thin-tailed (Gökçeada, Karayaka and Kıvırcık) or are known to be hybridized with them or are in close vicinity of Dağlıç - Kıvırcık hybrids (Çine

Çaparı). Hence, the VRQ allele is present in the Western, Northern and Northwestern part of Turkey. Since European sheep are predominantly thin-tailed and the Turkish sheep carrying the VRQ allele are distributed relatively close to Europe, they may had common origin with the European sheep in the recent past and as a consequence of having common gene pool, they might have possessed the allele. The following observations were also confirming that Kıvırcık, Gökçeada and Karayaka breeds are highly resembling to European sheep breeds. For instance, the rare VRH allele was found only in Kıvırcık breed in the present study. This allele, VRH, was firstly detected in Romanian Turcana sheep (Coşier *et al.,* 2011). Kıvırcık was proposed to be brought from Romania (Draganescu, 2007), hence, the former observation supports the proposition.

The AHR allele was found only in Gökçeada breed, which was firstly identified at low frequencies in European sheep breeds, such as Texel, Nolana and Suffolk sheep (Kutzer *et al.,* 2002), while another rare allele, VRR, was firstly observed in the same study (Kutzer *et al.,* 2002), which was only found in Karayaka breed in this study. Later, this allele was observed in 21 sheep breeds which were raised in the United States of America (DeSilva *et al.,* 2003). These observations may also indicate the presence of a common gene pool of Turkish and European sheep.

On the other hand, it was observed that Asian sheep have lower frequencies for the most classical scrapie-susceptible allele, VRQ compared with European sheep (Tsunoda *et al.,* 2010**;** Karami *et al.,* 2011; Guan *et al.,* 2011). Asian sheep predominantly have fat-tail and they harbor mtDNA haplogroups A, B and C (HPG-A, B and C) compared to mainly mtDNA haplogroup B (HPG-B) of European sheep (Bruford and Towsend, 2006; Tapio *et al.,* 2006). Y-chromosome dependent haplotypes also differ between the sheep of these two continents. Asian sheep is characterized by the H4 Y-chromosome haplotype, whereas the European sheep is characterized by H5 Y-chromosome haplotype (Meadows and Kijas, 2009), still it must be noted that these haplotypes are region spesific but they are in

low frequencies (e.g., H4 (0,007) and H5 (0,104) in Meadows *et al.,* (2006) and H4 (0,005) and H5 (0,078) in Meadows and Kijas, 2009). These difference in frequencies may indicate the existence of two different gene pools for Asian and European sheep, where the different frequencies of the VRQ allele are another manifestation of these two distinct gene pools. Turkey being a bridge between the two continents seemed to harbor sheep from both gene pools. As well as observing the VRQ allele in thin-tailed breeds, the existence of Asian gene pool as evidenced by observing H4 Y-chromosome haplotype only in fat-tailed sheep (Oner *et al.,* 2010) and the detection of a rare allele, TRQ, which is present in Asian (Guan *et al.,* 2011*)* and in Turkish sheep, but not in sheep of Europe supported this argument. Furthermore, presence of two different gene pools for the European and Asian sheep and the observation of slightly higher frequencies of the VRQ allele among the European sheep fits with the higher incidence of classical scrapie in Europe.

In addition to these commonly seen alleles, some rare alleles, such as AHH, ARK, THQ, VRR, AHR, TRH and VRH, were also found in Turkish sheep investigated. Some of these rarely observed alleles were seen in previous studies, whereas some of them were absent in previous studies of Turkish sheep. For instance, in the present study, the allele AHH was observed in Morkaraman sheep, which was previously detected in Sakız breed in another study from Turkey (Elmacı *et al*., 2009) and also in Chinese merino (Chen *et al*., 2010), whereas the allele TRH (0,023) was found only in Karayaka in the current study, which was previously detected in Sakız breed in Elmacı *et al.* (2009). Alleles THQ, VRR, AHR and VRH that were observed in this study were not previously detected in Turkish sheep.

In the current study, the alleles ARR, ARQ and ARH, were observed most commonly in all sheep breeds. These observations are in good fit with the results of some studies from European and Asian countries, such as Germany (Lipsky *et*

al., 2008), the Netherlands (Hagenaars *et al.,* 2010), Portugal (Gama *et al.,* 2006), Romania (Otelea et al., 2011), Spain (Alvarez *et al.,* 2007), China (Lan et al., 2006), Mongolia (Wang *et al.,* 2008), Iran (Karami *et al.,* 2011) and Pakistan (Babar *et al.,* 2009) as can be seen both from Table 4. 1 and Figure 4. 1.

Additionaly, other two rare alleles, the ALQ and TRR, which were not found in Turkish sheep breeds, was detected in European sheep at very low frequencies. The rare ALQ allele was observed only in a study from Spain (Alvarez *et al.,* 2006), whereas the allele TRR was observed only in Romania (Constantinescu *et al.,* 2010). At the same time, this rare allele, TRR, was also found in China (Guan *et al.,* 2011). The occurrence of these rare alleles on the edges of the world might be the result of the new mutations in ovine *PrP* gene. On the other hand, it is also possible that there might be a link between Chinese and Romanian sheep.

Table 4. 1 Allele frequencies of classical scrapie in different sheep populations from different countries around the world and in Turkey. Table adapted from the Final Project Report: Surveillance of prion protein gene polymorphisms in Pakistani and Turkish native sheep breeds (TUBITAK –MOST – Project no: 108O708).

Country (# of breeds)	PrP Allele Frequencies										Reference
ASIA											
	ARR	**ARQ**	**ARH**	**AHQ**	**VRQ**	**TRQ**	**ALQ**	**ARK**	**TRR**	**TRK**	
Pakistan (9)	0.060	0.750	0.160	0.030	-	-	-	-	-	-	Babar *et al.*. 2008
Pakistan (9)	0.065	0.733	0.167	0.025	-	-	-	-	-	-	Babar *et al.*. 2009
Iran (1)	0.216	0.678	0.100	-	0.006	-	-	-	-	-	Karami *et al.*. 2011
China (1)	0.090	0.752	0.126	0.023	-	-	-	0.009	-	-	Lan *et al.*. 2006
China (1)	0.183	0.577	0.097	0.005	0.002	0.066	-	0.058	0.005	0.002	Guan *et al.*. 2011
China (2)	0.067	0.866	-	-	-	-	-	0.067	-	-	Han *et al.*. 2011
China (3)	0.081	0.842	0.077	-	-	-	-	-	-	-	Tsunoda *et al.*. 2010
Mongolia (4)	0.158	0.777	0.038	0.011	0.014	-	-	0.001	-	-	Gombojav *et al.*. 2003
Mongolia (3)	0.096	0.844	0.044	-	-	-	-	0.015	-	-	Wang *et al.*. 2008
Mongolia (1)	0.132	0.770	0.086	0.006	0.006	-	-	-	-	-	Tsunoda *et al.*. 2010
Vietnam (1)	-	1.00	-	-	-	-	-	-	-	-	Tsunoda *et al.*. 2010
Nepal (4)	0.005	0.901	0.079	0.015	-	-	-	-	-	-	Tsunoda *et al.*. 2010
Myanmar (1)	0.007	0.968	0.013	-	0.013	-	-	-	-	-	Tsunoda *et al.*. 2010
Bhutan (3)	0.042	0.917	0.010	-	0.031						Tsunoda *et al.*. 2010
Kuwait (1)	0.167	0.524	0.310	-	-	-	-	-	-	-	Tsunoda *et al.*. 2010
EUROPE											
	ARR	**ARQ**	**ARH**	**AHQ**	**VRQ**	**TRQ**	**ALQ**	**ARK**	**TRR**	**TRK**	
Germany (5)	0.343	0.556	0.013	0.075	0.010	-	-	-	-	-	Drögemüller *et al.*. 2001
Germany (9)	0.376	0.483	0.020	0.116	0.007	-	-	-	-	-	Lipsky *et al.*. 2008
Germany (2)	0.168	0.727	0.001	0.101	0.002						Lühken *et al.*. 2004
Austria (4)	0.207	0.680	0.029	0.074	0.010	-	-	-	-	-	Sipos *et al.*. 2002
Bulgaria (8)	0.354	0.528	0.014	0.068	0.048	-	-	-	-	-	Sirakov *et al.*. 2011
France (29)	0.426	0.493	-	0.028	0.053	-	-	-	-	-	François *et al.*. 2003
Netherlands (1)	0.479	0.428	-	0.028	0.065	-	-	-	-	-	Hagenaars *et al.*. 2010

Table 4. 1 Continued

Country (# of breeds)	PrP Allele Frequencies										Reference
	EUROPE										
	ARR	ARQ	ARH	AHQ	VRQ	TRQ	ALQ	ARK	TRR	TRK	
England (11)	0.499	0.312	0.070	0.085	0.036	-	-	-	-	-	Roden *et al.*. 2006
England (4)	0.679	0.209	0.018	0.083	0.009	-	-	-	-	-	Boulton *et al.*. 2010
England (3)	0.158	0.547	0.053	0.165	0.077						Tsunoda *et al.*. 2010
Scotland (2)	0.312	0.601	-	0.077	0.010	-	-	-	-	-	Sawalha *et al.*. 2007
Italy (3)	0.348	0.562	0.019	0.039	0.030	-	-	-	-	-	Van Kaam *et al.*. 2008
Italy (1)	0.083	0.744	0.041	0.038	0.068	-	-	0.025	-	-	Acutis *et al.*. 2004
Italy (2)	0.401	0.577	0.001	0.020	-	-	-	-	-	-	Pongolini *et al.*. 2009
Italy (1)	0.024	0.658	0.023	0.041	0.043	-	-	-	-	-	Mazza *et al.*. 2010
Portugal (16)	0.273	0.646	0.014	0.028	0.037	-	-	-	-	-	Gama *et al.*. 2006
Poland (4)	0.352	0.633	-	-	0.015	-	-	-	-	-	Wisniewska *et al.*. 2006
Poland (4)	0.624	0.317	-	0.027	0.031	-	-	-	-	-	Wisniewska *et al.*. 2009
Romania (9)	0.251	0.625	0.019	0.022	0.157	-	-	-	-	-	Otelea *et al.*. 2011
Romania (1)	0.354	0.540	0.006	0.030	0.060	-	-	0.0079	0.0017	-	Constantinescu *et al.*. 2010
Slovenia (1)	0.174.	0.632	0.083	0.074	0.037	-	-	-	-	-	Gorjanc *et al.*. 2010
Slovenia (2)	0.186	0.655	0.045	0.083	0.031	-	-	-	-	-	Kastelic and Kompan. 2009
Greece (2)	0.110	0.788	0.006	0.058	0.026	-	-	0.002	-	-	Ekateriniadou *et al.*. 2007
Spain (2)	0.254	0.708	-	0.004	0.033	-	-	-	-	-	Hurtado *et al.*. 2002
Spain (1)	0.185	0.752	0.013	0.023	0.002						Ponz *et al.*. 2006
Spain (13)	0.199	0.694	0.035	0.028	0.045	-	-	-	-	-	Acin *et al.*. 2004
Spain (2)	0.216	0.700	0.042	0.027	0.013	-	0.002	-	-	-	Alvarez *et al.*. 2006
Spain (1)	0.227	0.737	0.020	0.002	0.012	-	-	-	-	-	Alvarez *et al.*. 2007
Spain (3)	0.292	0.619	0.030	0.015	0.044	-	-	-	-	-	Alvarez *et al.*. 2009

Table 4. 1 Continued

Country (# of breeds)	PrP Allele Frequencies													Reference
	TURKEY													
	ARR	**ARQ**	**ARH**	**AHQ**	**VRQ**	**TRQ**	**ALQ**	**ARK**	**TRR**	**TRK**				
Turkey (5)	0.140	0.710	0.065	-	0.015	0.045	-	0.025	-	-				Alvarez *et al.*. 2011
Turkey (3)	0.320	0.450	0.120	0.060	0.012	0.010	-	0.001	-	-				Oner *et al.*. 2011
Turkey (4)	0.181	0.710	0.095	0.008	0.004	-	-	-	-	-				Lühken *et al.*. 2008
Turkey (3)	0.400	0.500	0.020	0.005	0.017	0.030	-	-	-	-				Ün *et al.*. 2008
Turkey (2)	0.060	0.770	0.100	-	0.011	0.050		0.005						Frootan *et al.*. 2011
	ARR	**ARQ**	**ARH**	**AHQ**	**VRQ**	**TRQ**	**AHH**	**ARK**	**THQ**	**VRR**	**AHR**	**TRH**	**VRH**	
Turkey (14)	0.194	0.609	0.129	0.023	0.010	0.021	0.001	0.008	0.001	0.001	0.002	0.002	0.001	This study. 2012

Lastly, the allele THQ (0,012), which was only found in Sakız breed in the current study, is a novel allele and never detected in any sheep breed in any study before. Since Sakız has relatively narrower geographical distribution compared to other Turkish breeds and more over, Meadows and Kijas, (2009) reported that it has the H12 Y-chromosome haplotype, which is unique, support the proposition that Sakız is a distinct breed. Moreover, since Sakiz breed is located in the Western part of Turkey (close to Europe), it might be expected to have the most classical scrapie-susceptible VRQ allele. However, the VRQ allele was not present in this breed. The VRQ allele which was at a low frequency might have been lost due to random genetic drift.

Figure 4. 1, given below, shows the distribution of classical scrapie allele frequencies in Europe, Asia and Turkey.

On the contrary to many studies about classical scrapie genotyping, there exist very few countries (France, Norway (Moum *et al.,* 2005), Germany, England (Benestad *et al.,* 2008) and New Zealand (Bossers *et al.,* 1999) where the alleles and genotypes associated with atypical scrapie were recorded in sheep. The frequency of most atypical scrapie-susceptible alleles, AFRQ and ALHQ in these studies vary (Table 4. 2). For instance, the AFRQ allele was observed at a frequency of 0,136 in Norway (Moum *et al.,* 2005), whereas it was found at a much lower frequency (0,025) in New Zealand (Bossers *et al.,* 1999). Although the most atypical scrapie-susceptible alleles were detected at relatively low frequencies in sheep around the world, some countries (e.g., New Zealand (Kittelberger *et al.,* 2010), United Kingdom (Simmons *et al.,* 2010) and Spain (Rodriguez-Martinez *et al.,* 2010) continued to report atypical scrapie cases. To give an example, atypical scrapie cases were found in Canadian sheep with the ALRQ/ALRR genotype. This genotype is known to be resistant to classical scrapie (Hunter *et al.,* 2007), presence of sheep with this genotypes, which are relatively resistant to classical scrapie however highly susceptible to atypical scrapie

(Benestad *et al.,* 2003), are probably the reason for detecting atypical cases in Canada. Furthermore, within the context of previous breeding programs that aim to control classical scrapie, observation is supporting the argument that the frequency of classical scrapie-resistant genotypes might have been increased at the expense of increased atypical scrapie-susceptibility in sheep populations.

In summary, one novel allele ($TL_{141}HQ$) associated with atypical scrapie was observed in Sakız breed in this study. Additionally, the frequency of most atypical scrapie-susceptible alleles, AFRQ (0,002) and ALHQ (0,023), was very low in Turkish sheep breeds compared to previous studies in Europe and New Zealand. Because of the very limited reports covering the detection of alleles associated with atypical scrapie in both Turkish sheep (Alvarez *et al.,* 2011) and in sheep from other countries, the present study can be considered as one of the pioneer studies in reporting a novel allele and frequencies of atypical scrapie alleles/genotypes in the world.

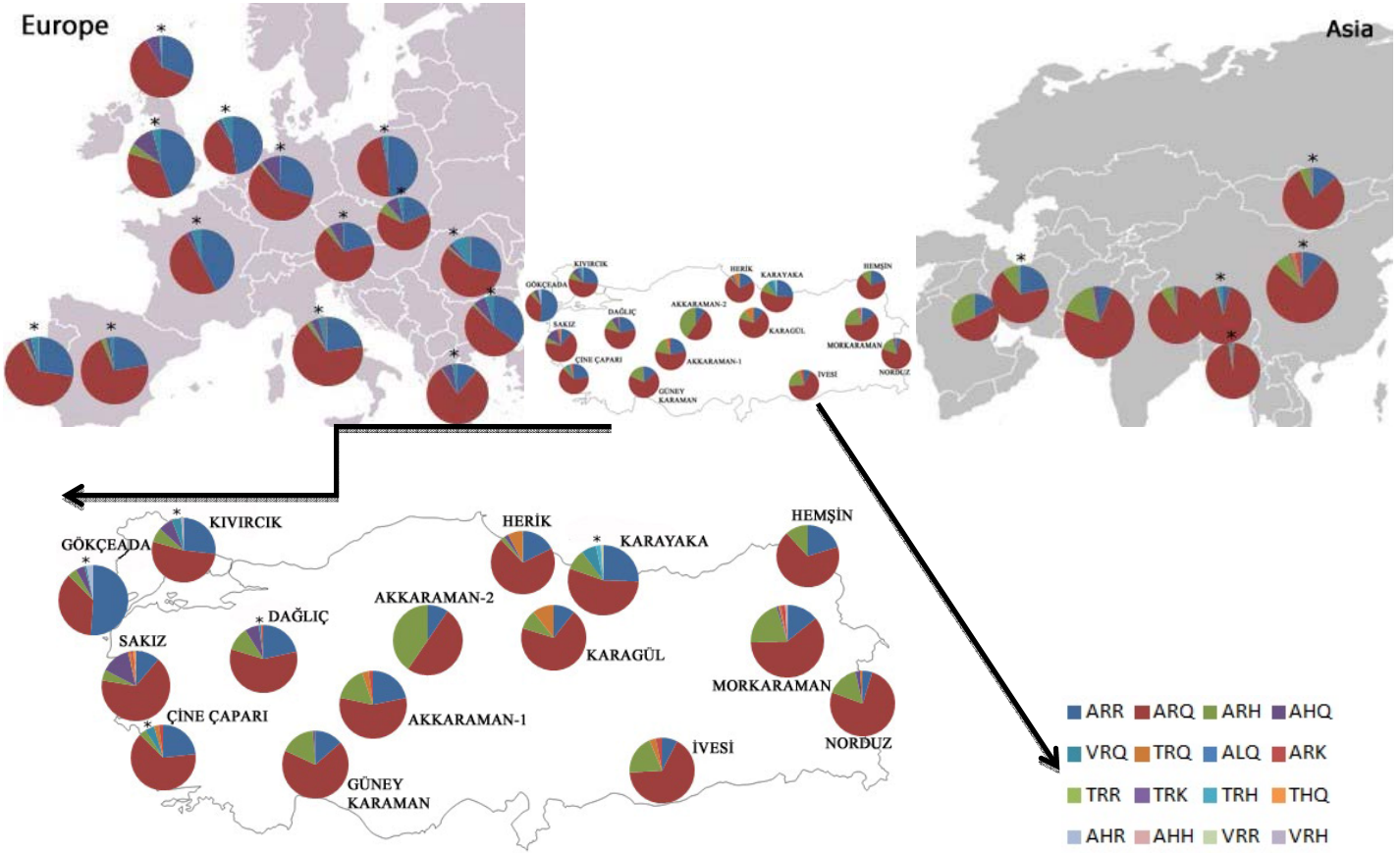


Figure 4. 1 The distribution of classical scrapie allele frequencies in Europe, Asia and Turkey. "*" represents the most classical scrapie-susceptible VRQ allele.

The distribution of frequencies of alleles associated with atypical scrapie in Europe and New Zealand were presented in Table 4. 2.

Besides, Figure 4. 2 showed the atypical scrapie allele frequencies in Turkey to be compared with the frequencies in Europe and New Zealand.

According to observations associated both with classical and atypical scrapie haplotypes, Turkey was a hot spot for observing PrP haplotypes since Turkish sheep are found to have the highest PrP genetic variability with 13 alleles associated with classical scrapie and 14 alleles associated with atypical scrapie compared to both previous studies from Turkey and from other countries. For instance, 9 alleles for classical scrapie were found in Chinese sheep (Guan *et al.,* 2011) and 7 alleles also for classical scrapie were detected in Romania (Constantinescu *et al.,* 2010), whereas 5 alleles associated with atypical scrapie were observed in European sheep previously. Previous studies suggest that Turkey is subjected to migration of sheep from all directions (Peters *et al.,* 1999) since the domestication of sheep. So, the highest genetic variability in *PrP* gene of Turkish sheep might be explained by the expectation of high genetic diversity in Turkish sheep which is subjected to migration from all directions and their enriched gene pools by the migration of sheep (Bruford *et al.,* 2003; Uzun *et al.,* 2006; Meadows *et al.,* 2007; Togan *et al.,* 2007). Moreover, lack of scrapie selection programmes for *PrP* gene might have contributed for the Turkish sheep to maintain its high PrP genetic diversity.

Table 4. 2 Allele frequencies of atypical scrapie in European sheep, sheep from New Zealand and Turkish native sheep. Table adapted from the Final Project Report: Surveillance of prion protein gene polymorphisms in Pakistani and Turkish native sheep breeds (TUBITAK –MOST – Project no: 108O708).

Country	Frequencies of alleles associated with Atypical Scrapie														Reference
EUROPE															
	ALRR	**ALRQ**	**ALRH**	**ALHQ**	**AFRQ**	**VLRQ**									
France, Norway, Germany, England	0.237	0.168	0.012	0.310	0.261	0.012									Benestad *et al.*, 2008
Norway	0.239	0.301	0.032	0.218	0.136	0.074									Moum *et al.*, 2005
NEW ZEALAND															
New Zealand	0.471	0.407	-	0.071	0.025	0.025									Bossers *et al.*, 1999
	TURKEY														
	ALRR	**ALRQ**	**ALRH**	**ALHQ**	**AFRQ**	**VLRQ**	**TLRQ**	**ALHH**	**ALRK**	**TLHQ**	**VLRR**	**ALHR**	**TLRH**	**VLRH**	
Turkey	0.194	0.609	0.125	0.023	0.002	0.010	0.021	0.001	0.009	0.001	0.001	0.002	0.002	0.001	This study, 2012

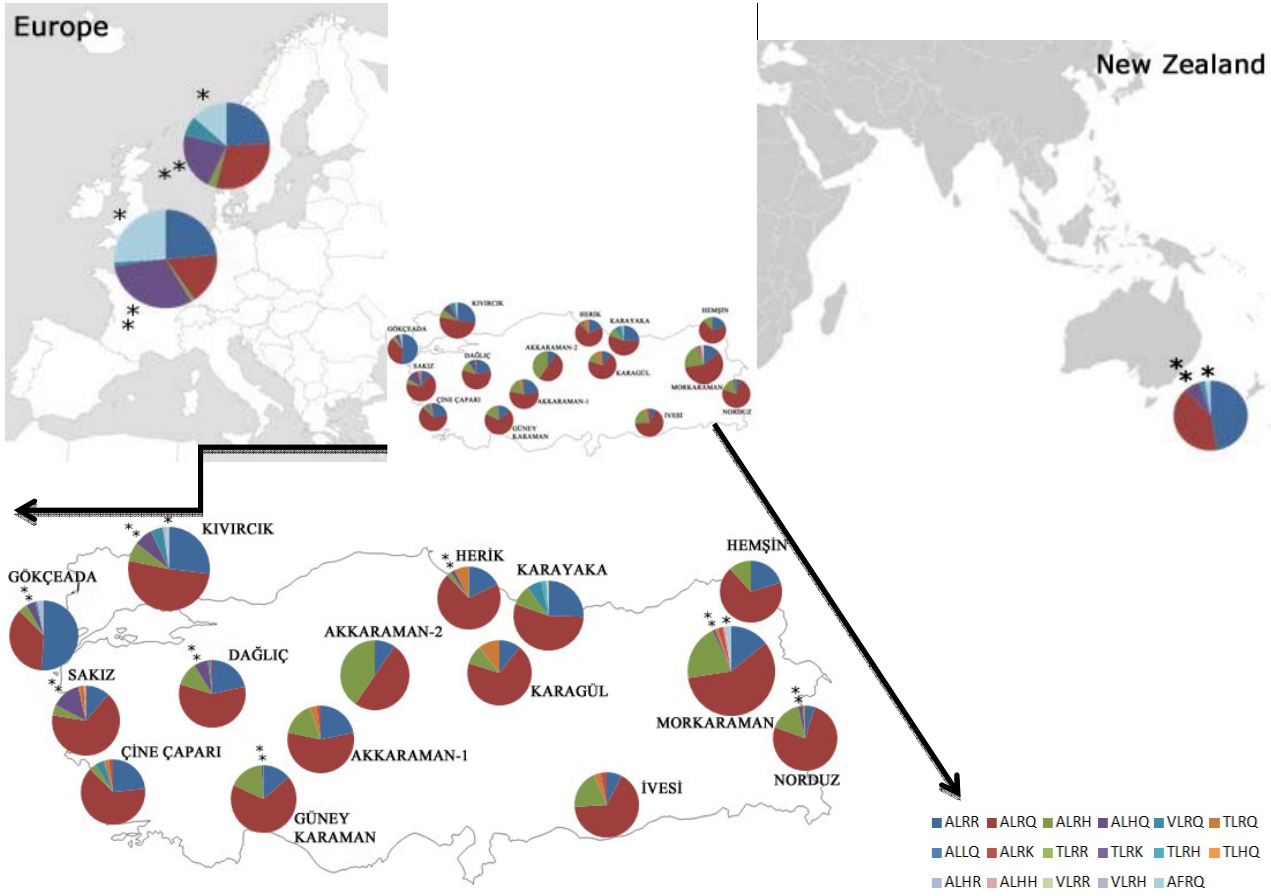


Figure 4. 2 The distribution of atypical scrapie allele frequencies in Europe, New Zealand and Turkey. Stars represent the most atypical scrapie-susceptible alleles. "*" refers to the AFRQ allele, whereas "**" refers to the ALHQ allele.

4. 2 Sampling Effects in Turkish Sheep Breeds

4. 2. 1 The effect of Different Sampling Time and Location

As also mentioned above, some alleles, such as the wild-type allele, ARQ and the most classical scrapie-resistant allele, ARR were observed at similar frequencies in the present study and in previous studies of Turkish sheep breeds. However, some other alleles detected in breeds studied in the current study were absent even in the same breeds investigated in previous studies of Turkish native sheep. For this reason, some comparison between Turkish sheep was done according to their allele frequencies of classical scrapie to suggest some explanations for this observation.

For instance, despite the fact of not observing the most classical scrapie-susceptible allele, VRQ, in Hemşin breed in the present study, it was found in the same sheep breed (0.043) in Alvarez et al. (2011). Moreover, the VRQ allele was present in Morkaraman sheep in both Alvarez et al. (2011) and Frootan et al. (2011), whereas it was absent in Morkaraman breed analyzed both in the current study and in Lühken et al. (2008). It can also be observed that the VRQ allele was detected in Ivesi breed in Frootan et al. (2011), whereas Ivesi sheep investigated in the present study did not exhibit this most classical scrapie-susceptible allele. Furthermore, the frequencies of alleles were detected in varying values in Akkaraman sheep which is represented by two independent samples sampled in two different times: in 2000 and in 2007.

In the current study, two independent populations of Akkaraman sheep belonging to same breed were analyzed. Akkaraman-1 population was sampled around Konya in 2007, while Akkaraman-2 was sampled earlier around Ankara, Konya and Sivas in 2000. It is seen that the allele ARK was only found in Akkaraman-1 sampled in 2007 but not in Akkaraman-2 population sampled in 2000, however,

this allele was present in Akkaraman breed in Alvarez et al. (2011). Also, the allele ARH was detected in Akkaraman sheep at very different frequencies. For instance, it was observed at a frequency of 0,163 in Akkaraman-1, whereas it was found at a 4 times higher frequency in Akkaraman-2 population in the present study. This allele was also observed in Morkaraman sheep at a frequency of 0,204 in the current study, whereas it was detected at a much more lower frequency of 0,015 again in Morkaraman breed in a different study (Alvarez et al. (2011).

First of all, it was observed that the presence of the major alleles (the allele with the maximum frequency, the second and the third most commonly seen alleles) are reproducible in repeated studies. Differences with respect to presence or absence of other alleles in low frequencies can be expected because they are subject to sampling the most. However, the allele ARH in Akkaraman populations is not a very low frequent allele since it was detected at a frequency of 0,163 in Akkaraman-1 and 0,403 in Akkaraman-2. Here, it is important to note that there is no isolation among the Anatolian breeds. For this reason, the sheep breeds are most probably highly admixed (Acar, 2010; Acan, 2012) and open to uncontrolled admixture. The nonhomogenous genetic structure of Turkish sheep breeds might explain these differences in allele frequencies of both independent samples of the same breed sampled in different locations and different times.

When all these observations were considered, it can be concluded that the distribution and prevelance of alleles associated with classical scrapie is dependent on both time and locations of sampling of sheep. However, the general trends of high ARQ allele frequency and the presence of VRQ allele and the ALHQ and AFRQ alleles, which are associated with high susceptibility to atypical scrapie, in thin-tailed Turkish sheep seem to be robust.

4. 2. 2 Results of the present study in relation to Hardy-Weinberg Equilibrium tests and Comparative Studies

It was also observed that the distribution of both classical and atypical genotypes differed from the Hardy–Weinberg equilibrium for all sheep breeds investigated in the present study. Similar significant deviations were obtained in Un et al. (2008) and Oner et al. (2011). One can conclude from these results that Turkish sheep breeds may have many subgroups that are isolated from each other (Wahlund Effect). Besides, because of using very few number of rams in breeding, sheep in general may have been suffering from small effective population size (N_e).

Furthermore, in the current study, both pairwise F_{ST} values (Excoffier *et al.,* 2006) and Nei's genetic distance (D_A) values (Nei, 1977) were estimated according to polymorphisms at 4 codons (136, 141, 154 and 171) to determine the genetic relation and differentiation among sheep populations. Moreover, the significance levels for pairwise F_{ST} values were observed.

When these pairwise F_{ST} values are considered, it was observed that Gökçeada breed was significantly differentiated from other populations in all of the pairwise comparisons, whereas Akkaraman-2 population was also relatively distinct from other populations but was not significantly different from Akkaraman-1. Besides, Nei's genetic distance (D_A) values also supported this observation since the values showed that the most divergent breeds were Gökçeada breed and Akkaraman-2 population. The differentiation of Akkaraman-2 population from other populations might be explained by its earlier sampling time compared to other breeds studied. This indicates that sampling time of sheep breeds might be effective on the genetic differentiation of breeds. Moreover, the separation of Gökçeada breed can be explained by high degree of genetic drift due to the isolation and presence of its small population size. The genetic drift is considered as the random change in the frequency of alleles within a population's gene pool. It can cause the frequency of

haplotypes to change in one direction or another (Allendorf and Luikart, 2007). Therefore, the differentiation of Gökçeada according to allele frequencies might be due to random genetic drift.

Furthermore, two neighbor joining (NJ) trees were constructed by using pairwise F_{ST} values and Nei's genetic distance (D_A) values in the present study. According to these NJ Trees, there were two distinct groups. Most of the breeds were together in one group, whereas Gökçeada was separated from other breeds in the second group. Togetherness of Akkaraman-1 and Akkaraman 2 populations in NJ Trees were another manifastation of the results of pairwise F_{ST} values.

Although neighbor joining (NJ) trees show the relationship between populations only in one dimension, Principal Component Analysis (PCA) allows to visualize this relationship in multiple dimensions. The PCA was constructed again according to polymorphisms at 4 codons (136, 141, 154 and 171), and it was also observed that most breeds (IVE, GK, MOR, AKK-1, DAG, KRY, KIV, CIC, HEM, SAK, KRG) grouped together in the center suggesting that they are genetically similar according to their allele frequencies associated both with classical and atypical scrapie. The separation of Gökçeada breed and Akkaraman-2 population from other breeds were also observed in the PCA. Neighbor joining (NJ) trees confirmed the previous inferences indicating that Akkaraman-2, which was sampled in 2000, might have been affected both by genetic drift and market economy over the years. And these effects might have resulted in its genetic structure. In addition, overall, Gökçeada population was observed to be distinct among the examined populations. As an explanation of the distinctness of Gökçeada, one can propose that Gökçeada being an island population could have been strongly affected by random genetic drift.

Most of sheep breeds are found to be genetically similar according to polymorhisms at codons 136, 141, 154 and 171. This genetic similarity between

breeds might be explained by the presence of few number of mutations, which may not be sufficient to separate the breeds, at these analyzed codons in the analysis.

4. 3. Distribution of Genotype Frequencies and their Risk Groups

It can also be seen in the results part that in the current study, the genotypes both associated with classical and atypical scrapie were identifed in Turkish native sheep. It was observed that overall, the most frequent genotype was ARQ/ARQ (0,498) which belongs to R3 risk group of classical scrapie. Similar result can also be observed in some previous studies of Turkish native sheep (Alvarez *et al,* 2011; Frootan *et al.,* 2011) and many other studies from other countries (Sipos *et al.,* 2002; Acin *et al.,* 2004; Lipsky *et al.,* 2008; Kipanyula *et al.,* 2009; Tsunoda *et al.,* 2010). The second most frequent genotype, ARR/ARQ, which is one of the classical scrapie-resistant genotypes belonging to R2 risk group, was present in all fourteen breeds. This observation also supports the results of Alvarez et al. (2011). However, it was the most commonly seen genotype in Un et al. (2008) and Oner et al. (2011). Moreover, the VRQ/VRQ genotype, which is highly associated with susceptibility to classical scrapie and belong to the highest classical scrapie risk group (R5), was detected at low frequencies in three sheep breeds studied in the present study supporting the results of Frootan et al. (2011) and Oner et al. (2011). On the other hand, this genotype was absent in Turkish sheep investigated in Un et al. (2011) and Alvarez et al. (2011).

In the light of above observations, it was established that the majority of genotypes in Turkish sheep breeds belong to third (R3) classical scrapie risk group, that with intermediate risk of classical scrapie. However, Un et al. (2008) reported Turkish sheep with most of the genotypes belong to classical scrapie-resistant risk groups (R1 and R2). Moreover, Oner et al. (2011) suggested that the genotypes associated with classical scrapie belonged to R2 and R3 risk groups in Turkish sheep. In

general, it can be concluded that Turkish native sheep breeds have intermediate classical scrapie risk. Furthermore, since Turkish sheep breeds have relatively low frequencies of classical scrapie-susceptible alleles and genotypes, whereas they have higher frequencies of alleles and genotypes associated with higher classical scrapie-resistance, not diagnosing classical or atypical scrapie cases in Turkish native sheep is not surprising. Another reason could be that Turkish sheep did not come across with a scrapie agent. Moreover, in Turkey feeds containing animal tissue are not used. Perhaps, the usage of feed without animal tissue is probably the most significant reason for not observing scrapie cases in Turkish sheep.

Turkish sheep breeds can be classified as having low and high risks of classical scrapie in the present study. According to genotypes that were classified in risk groups, Akkaraman, Güney Karaman, Karagül, Hemşin and Ivesi breeds were found to have realtively high frequencies of classical scrapie-resistant genotypes, whereas Kıvırcık, Dağlıç, Gökçeada, Çine Çaparı and Karayaka breeds were found to have classical scrapie-susceptible genotypes. The breeds, that are found to have realtively high classical scrapie risk, are known to be located on the west of Turkey except Karayaka breed. It might be argued that if a national scrapie program is going to be started in the country, genetic management can be applied to those breeds under the relatively high classical scrapie risk. It is also known that high rainfall is one of the important characteristics of the Black Sea Region of Turkey (Hay *et al.,* 1991). Since it was previously reported that high mean annual rainfall might be one of the environmental risk factors for classical scrapie (Stevens *et al.,* 2009), Karayaka breed which is located in the Black Sea Region, perhaps, must have the highest priority in management. Moreover, some significant correlations were found between soil geochemistry and scrapie (Imrie *et al.,* 2009) and soil drainage is found to be significantly associated with the occurrence of the disease (Stevens *et al.,* 2009). If these findings can be generalized, the distribution of soil geochemistry parameters would also be very

usefull in the prioritization of the breeds for the management efforts. However, there is no enough information about the characteristics of soil in Turkey.

In addition to all alleles observed for 3 site (classical scrapie), all alleles previously observed for 4 site (atypical scrapie) were also recorded in Turkish sheep in the present study. It was previously reported that the alleles $AL_{141}HQ$ (highest risk category) and $AF_{141}RQ$ (highest risk category), presumably related with high susceptibility to atypical scrapie (Moum *et al.,* 2005), are found in some of the sheep breeds studied in this study. The ALHQ allele was detected in eight of fourteen breeds at low frequencies (0,010-0,137), whereas the AFRQ allele was present only in two of the breeds also at low frequencies (Kıvırcık (0,012) and Morkaraman (0,020)). The most atypical scrapie-susceptible allele, AFRQ, was previously detected only in Tuj breed (0.059) in Turkey (Alvarez *et al.,* 2011). Considering above observations, it can be concluded that Turkish sheep breeds have relatively low frequencies of atypical scrapie-susceptible alleles compared to previous studies in Europe and New Zealand.

Furthermore, it was observed that the most frequent genotypes associated with atypical scrapie belong to zero (0) and one (1) atypical risk groups in Turkish sheep in the present study and concluded that Turkish sheep breeds can be considered to have higher frequencies of atypical scrapie-resistant genotypes, hence, low atypical scrapie risk in general. Since no atypical scrapie cases were detected in Turkish sheep so far, the reason for this may be explained by Turkish sheep breeds having low frequencies of atypical scrapie-susceptible alleles, ALHQ (0,023) and AFRQ (0,002)), and higher frequencies of atypical scrapie-resistant alleles in comparison with the previous studies in European (Benestad *et al.,* 2008; Moum *et al.,* 2005) and New Zealand sheep (Bossers *et al.,* 1999). Another reason for not detecting atypical scrapie cases in Turkish sheep might be the presence of early lamb slaughtering in Turkey, especially in regions of Marmara, Eegean, Central Anatolia and Black Sea (Karacasu, 1974). Since it is known that atypical

scrapie occurs in older sheep (Lühken *et al.,* 2007; OIE *Terrestrial Manual,* 2009), slaughtering sheep at very early ages in Turkey may be the other reason for not diagnosing atypical scrapie in Turkish sheep breeds.

As a conclusion, the outcomes of the present study allow us to understand the distribution of both classical and atypical scrapie susceptibility in Turkish native sheep breeds, hence, fill the gap which is present in the data of geographic distribution of *PrP* gene polymorphisms in the world. The data from the current study may help to establish a breeding program for classical scrapie control in the country, and to have fully classical scrapie-resistant breeds in the future. Since atypical scrapie has been found at low incidence rates in a number of European countries (Fediaevsky *et al.,* 2009; Green *et al.,* 2007) and Turkish sheep is found to have very low atypical scrapie risk, it can be concluded that there is no need for an urgent breeding programme for atypical scrapie control in Turkey. However, as a result of classical scrapie control programmes, achieving sheep populations with the most classical scrapie-resistant genotypes may lead to populations to have high atypical scrapie-susceptibility. For this reason, Turkish sheep is needed to be monitored carefully for atypical scrapie.

In the present study, gene bank codes for some individuals that are males and have to be used in breeding in order to reduce the risk of classical scrapie were identified. Besides, codes for female individuals that must not be used in breeding since they carry the most classical scrapie-susceptible allele, VRQ, also in order to reduce the risk of classical scrapie in Turkey. Therefore, this data will be important and useful for the authorities responsible from the avoidance of scrapie in Turkey, for animal breeders associations and for the research units of universities.

By establishing and implementing scrapie control measures by the authorities, Turkey will also benefit economically. Above all, since it is found that there is a

link between scrapie and mad cow disease (BSE), which in turn may transmit the fatal variant Creutzfeld-Jacob disease (vCJD) to humans (Bruce *et al.,* 1997), this data will be beneficial for the public health in the country.

CHAPTER 5

CONCLUSIONS

In the present study, 15 populations from 14 native Turkish sheep breeds (Norduz, Çineçaparı, Dağlıç, Herik, Kıvırcık, Akkaraman (represented by two independent samples (Akkaraman-1 and Akkaraman-2)), Sakız, İvesi, Morkaraman, Hemşin, Karagül, Karayaka, Gökçeada and Güney Karaman; in total represented by 655 individuals) were examined with respect to their polymorphisms of *PrP* gene (at 4 different codons: 136, 141, 154 and 171).

The results and conclusions of the present study can be listed as follows:

1) The alleles ARR, ARQ and ARH, were detected most commonly in all Turkish sheep breeds analyzed. This result is in good fit with the results of some other studies from European and Asian countries.

2) The most frequent allele was the ARQ allele, which is one of the classical scrapie-susceptible alleles, followed by ARR (0,194) and ARH (0,129) alleles in Turkish sheep. These results support the hypothesis that the allele ARQ is the wild-type allele in sheep.

3) The most classical scrapie-resistance allele, ARR, was found in all of the sheep breeds at frequencies ranging from 0,048 in Norduz to 0,510 in Gökçeada.

4) The most classical scrapie-susceptible VRQ allele was detected in 5 of the breeds (Çine Çaparı, Dağlıç, Kıvırcık, Karayaka and Gökçeada) with relatively

low frequencies. Overall, it was found at a low frequency of 0,010. In addition, the most classical scrapie-susceptible VRQ/VRQ genotype was observed in Kıvırcık, Çineçaparı and Karayaka at a very low frequency of 0,003 compared to Europe (0,007-0,129). These results indicate the detection of most classical-scrapie susceptible allele and genotype, VRQ and VRQ/VRQ, respectively, at very low frequencies in Turkish sheep compared to European sheep.

5) Some rarely found alleles, such as ALHH (0,001), ALRK (0,009), TLHQ (0,001), VLRR (0,001), ALHR (0,002), TLRH (0,002) and VLRH (0,001), were also found in Turkish sheep investigated but at low frequencies (0,001-0,009).

6) One novel allele, which is the $TL_{141}HQ$ allele, was observed only in Sakız breed in this study and never been detected in any sheep breeds in any other study before. Since it is reported that Sakız has the unique H12 Y-chromosome haplotype, this observation also supports the proposition that Sakız is a distinct breed.

7) Since ARQ allele was high and VRQ allele was observed in thin-tailed sheep (European like sheep), it is concluded that the general trends of high ARQ allele frequency and the presence of VRQ allele in sheep of Europe is repeated among the Turkish sheep.

8) The most atypical scrapie-susceptible alleles, ALHQ and AFRQ, were found in some of the sheep breeds examined. The ALHQ allele was detected in eight of the breeds (Norduz, Dağlıç, Herik, Kıvırcık, Sakız, Morkaraman, Gökçeada and Güney Karaman) with the range of frequencies 0,010 in Morkaraman and 0,137 in Sakız, while the AFRQ allele was present only in Kıvırcık (0,012) and Morkaraman (0,020) at low frequencies compared to previous studies in Europe (0,198) and New Zealand (0,025). For this reason, it is concluded that Turkish

sheep breeds have low frequencies of atypical scrapie-susceptible alleles in comparison with European and New Zealand sheep.

9) The presence of ALHQ and AFRQ alleles which are associated with high susceptibility to atypical scrapie is almost exclusively in thin-tailed Turkish sheep.

10) Since Turkish sheep are found to have the highest PrP genetic variability with 13 alleles associated with classical scrapie and 14 alleles associated with atypical scrapie compared to both previous studies from Turkey and from other countries, Turkey is probably a hot spot for observing PrP haplotypes. The reason is probably the expectation of high genetic diversity in Turkish sheep which is subjected to migration from all directions since the domestication of sheep and the lack of scrapie selection programmes for *PrP* gene in Turkey.

11) It is found that most of the genotypes associated with classical scrapie belong to third (R3) classical scrapie risk group in Turkish native sheep. Therefore, Turkish sheep breeds are found to have intermediate level of classical scrapie risk.

12) Kıvırcık, Dağlıç, Gökçeada, Çine Çaparı and Karayaka breeds are found to have classical scrapie-susceptible genotypes, in other words, they have higher risk of classical scrapie. If a scrapie control program is going to be applied in the country, genetic management can be applied to those breeds that are under the relatively high risk of classical scrapie. Particularly, Karayaka breed located in the Black Sea Region, perhaps, must have the highest priority in management, because of the assumption of high mean annual rainfall might be one of the environmental risk factors for classical scrapie.

13) Most of the genotypes associated with atypical scrapie are found to belong to zero (0) and one (1) atypical scrapie risk group. This indicate that Turkish sheep breeds are found to have very low atypical scrapie risk. However, Kıvırcık,

Gökçeada, Sakız, Dağlıç, Norduz and Morkaraman breeds are found to have atypical scrapie-susceptible genotypes, hence, they have relatively higher atypical scrapie risk. Since low incidences of atypical scrapie is reported in some sheep populations from the world and it is found that there is very low risk of atypical scrapie in Turkish sheep breeds, instead of a breeding programme for atypical scrapie control in Turkey, Turkish sheep is needed to be monitored rigorously for atypical scrapie.

14) Most of sheep breeds are found to be genetically similar according to polymorhisms at codons 136, 141, 154 and 171 in statistical analyses. However, Akkaraman- 2 population and in particular, Gökçeada breed is found to be different from others.

15) Gökçeada population is observed to be distinct among the examined populations in pairwise comparisons and multidimensional analyses. Since Gökçeada is an island population, which makes it an isolated breed, it might be strongly affected by random genetic drift. So, this differentiation is not surprising.

16) Akkaraman-2 population is also found to be differentiated from some other populations. Since, Akkaraman-2 was sampled earlier (in year 2000) compared to the sampling times of other populations (2007-2010), we might be witnessing the effects of genetic drift and market economy on the samples of the same breed.

17) Gene bank codes for some individuals who were deposited to National Gene Banks of Turkey are screened for their scrapie risk. Gene bank codes for males that can be recommended to be used and codes for females that are recommended not be used in breeding programs were recorded and presented.

18) This data covering the *PrP* gene polymorhisms of 14 Turkish sheep breeds allow us to understand the distribution of both classical and atypical scrapie risk in

Turkey. For this reason, this data is important and also useful for the authorities responsible from the risk management of the disease scrapie in Turkey, for also animal breeders association and for the research units of universities.

19) In addition, the outcomes of the present study, by displaying the genetic distribution of both classical and atypical scrapie susceptibility in Turkey, will fill the gap which is present in the geographic distribution data of *PrP* gene polymorphisms in Euro-Asia.

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