THE PUBLISHING HOUSE
OF THE ROMANIAN ACADEMY

AGRICULTURE
Research article

COMPARATIVE STUDY CONCERNING THE GENE POLYMORPHISM OF PRION PROTEIN (PRP) IN THE BOTOSANI KARAKUL AND PALAS MEAT BREED SHEEP

Gheorghe HRINCĂ[1] and Petru Gabriel VICOVAN[2]

[1]Research and Development Station for Sheep and Goat Breeding, Popauti-Botosani, Romania
[2]Research and Development Institute for Sheep and Goat Breeding, Palas-Constantza, Romania
Corresponding author: Gheorghe HRINCĂ, E-mail: ghrinca@yahoo.com

Accepted May 25, 2014

The study describes the genetic polymorphism at the PrP locus level (associated with susceptibility/resistance to scrapie of small ruminants) in Botosani Karakul sheep and Palas Meat Breed. Identifying the genetic variants of prion protein was achieved by the Real-Time PCR technique. The phenotypic expression, allelic spreading and genotypic structures at PrP locus were analyzed. Essential differences were found between the two sheep breeds in terms of the number of alleles PrP, their frequencies and phenotypic and genotypic configuration of the prion panel. The polymorphism at prion gene locus is determined by the existence of five alleles (ARR, ARQ, AHQ, ARH and VRQ). In the Botosani Karakul breed only three alleles have been found (ARR, ARQ and ARH), whereas in the Palas Meat Breed all five alleles have met. Consequently, the prionic polymorphism is more emphasized in the Palas Meat Breed than in the Botosani Karakul. In Botosani Karakul only six genotypes (of 10 possible) are found, while in Palas Meat Breed almost all the 15 genotypes are expressed, except the homozygotes ARH/ARH. At the PrP locus, the homozygozity is very well represented compared to heterozygosity in Botosani Karakul, while in Palas Meat Breed the heterozygosity is more widespread than homozygozity. The distributional characteristics of prionic genotypes determine as the practical frequencies to conform to expectations of Hardy-Weinberg law in the Palas Meat breed, instead the Botosani Karakul breed is in very significantly genetic disequilibrium at the PrP locus.

Key words: prion, scrapie, Real-Time PCR technique, sheep.

INTRODUCTION

Prions are infectious microscopic protein particles devoid of genetic material (of any type of nucleic acid), similar to viruses, which are considered to be capable of self-reproduction and which causes a group of neurodegenerative diseases characterized by profound alteration of the central nervous system. Prion replication (in which strings of amino acids are reproduced), stands as an exception from the central tenet of biology stating that only the nucleic acids, such as DNA, can self-reproduce. The prion replication mechanism is still not clearly understood. For these reasons, the prions are perceived as being a peculiar nature and having a unique biology, representing a real challenge for the scientific world. This is because the central dogma of biology postulates that the nucleic acids constitute the only heritable genetic material[15, 20, 29].

According to Prusiner considerations (which introduced the prion concept)[21], prions are considered the pathogenic agent of spongiform encephalopathies (*TSEs*) found especially to farm animals with major impact on their health[22, 23]. In small ruminants (sheep, goats, mouflon) this disease called scrapie[23].

All mammalian genomes analyzed provide a copy of the *PrP* gene. The genes coding for *PrP* or *PrP*-like protein were also detected in birds, reptiles, amphibians and fishes[18,24,28,30,31], but in these species the diseases caused by prions have not been described. While the structure and sequence of prion protein is highly conserved among mammalian

species, in other vertebrate classes its sequence is very different from mammals[24].

The genetics always provides to scientific research from the animal breeding field many and diversified instruments to achieve new biological creations: breeds, lines or hybrids of domestic animals through different crossing (especially the industrial ones). This status quo creates preconditions to amplify the protein polymorphism, inclusively of the prionic one, which would increase the opportunities for higher spread of scrapie in sheep populations. On the other hand, the traditional animal breeding with either random or less controlled crossbreedings have the same effect.

Due to the importance of this disease for animal husbandry, studies of molecular genetics relating to the prion protein were performed widely in the countries of Western Europe in which the industrial crossbreeding is practiced to create new sheep types with high productive performance (in particular for the meat production)[9]. In Romania these research topics heave a fairly recent history and took into account the Merino, Tsigai and Tsurcana breeds specialized mainly for meat or milk productions [7, 19]. In the Botosani Karakul breed (sheep specialized for the lamb pelt production) the approach is almost episodic in this regard[13, 14, 16].

In the context described, the paper has proposed genotyping the sheep belonging to the Botosani Karakul breed and Palas Meat Breed for the determinant locus of susceptibility to scrapie; this is because a genetic approach in this regard, coupled with coherent selection and breeding actions, represents the best way to eradicate this terrible disease

MATERIALS AND METHODS

The biological material necessary to achieve the proposed experiment consisted of two sheep populations: one (comprising 123 animals) belonged to the Botosani Karakul breed (from the *Research and Development Station for Sheep and Goat Breeding Popăuți-Botosani*) and the other (composed of 317 animals) has been part of the Palas Meat Breed (from the *Research and Development Institute for Sheep and Goat Breeding Palas-Constantza*).

To identify some genes susceptible to contract the scrapie disease, blood and brain (brain stem) samples were collected from which the DNA was extracted and isolated. The blood sampling was performed by jugular venipuncture in vacutainers (15 ml) with EDTA (5%) as an anticoagulant. For the extraction of brainstem segments from sheep brain a surgical method was used [14].

Isolation and purification of genomic DNA from blood is achieved using the kit "Genomic Wizard" (Promega), and the one from brain homogenates using the kit "High Pure PCR Template Preparation" (Roche), in compliance with manufacturer specifications[16]. In both situations the extraction of genomic DNA from cells was made with aid of saline solutions and its isolation was carried out in four stages[14].

The Real Time PCR method, whose principle is fluorimetric detection in real time, coupled with the melting curve analysis, was used for sheep genotyping at the prion protein locus. The PCR technique uses detection kits provided by Roche [25] that contain hybridization probes designed at the level of codons 136, 154 and 171 of the prion protein which allow the fluorimetric detection and evaluation of the PCR products according to Fluorescence Resonance Energy Transfer (FRET) phenomenon[26].

The Real-Time PCR reaction occurred in the Light Cycler 2.0 apparatus carrying out in four phases: pre-incubation, amplification, melting curve and cooling, each reaction phase being performed at a specific temperature and in a well-defined time [14].

Identification of the genetic variants at the PrP locus was performed by melting curve analysis of amplification products depending on the number, position and shape of the peaks of these curves.

To confirm the accuracy of Real Time PCR method some of the samples were analyzed by sequencing technique too. Its use is indicated because is a specific method that provide a more precise diagnosis. The alignment of the PrP gene sequence prevailing in the GenBank database and the sequences obtained from the analyzed samples was performed by means of the Clustal application within the BioEdit program[27].

The allelic and genotypic frequencies at the PrP locus were calculated, as well as the zygosity status (ratio between homozygosity and heterozygosity at this locus). The Hi^2 test (χ^2) was used to estimate the difference signification between the observed frequencies and the expected ones of PrP genotypes within each sheep breed, as well as the empiric differences between the two breeds regarding the distributions of prionic genotypes.

RESULTS AND DISCUSSION

Genetic determinism of prion protein. In the ovine species a number of polymorphisms have been identified at the PrP gene level but it has been demonstrated that only those located in the coding region are associated with susceptibility to scrapie. Most polymorphisms of sheep described so far are located in the C-terminal domain between codons 98 and 234. In the European sheep breeds several polymorphic codons were analyzed: 112, 136, 154, 137, 141, 138, 151, 168, 171, 175, 176, 180 and 211 [17, 19]. By combining, these polymorphisms generate 25 allelic variants of prion protein.

Of those 13 codons, only three have relevance regarding the association degree with resistance/ susceptibility to scrapie as follows: 136, 154 and 171[11]. These three codons are involved in the classical scrapie and most studies related to this issue. But in 1998, the spectrum of transmissible

spongiform encephalopathy in small ruminants was extended by the discovery in Norway sheep of an experimentally transmissible neurological disease that was clearly distinguishable from classical scrapie cases that had been reported so far and was therefore considered to be an "atypical" form of scrapie. The mutation for atypical scrapie occurs at codon 141. The atypical scrapie has been designed Nor98 scrapie or Nor98-like scrapie[2, 3]. Following its recognition in sheep, Atypical/Nor98 was detected in goats too [1]. Therefore, the aim of this study was to identify possible polymorphisms at the PrP gene locus within codons of the positions 136, 154 and 171 in the Botosani Karakul sheep and Palas Meat Breed.

The literature has described more than 15 alleles at the prion protein locus. However, of these, only five alleles are involved in characterizing and determining the level of resistance / susceptibility to scrapie in sheep: ARR, AHQ, ARH, ARQ and VRQ. The order of their enumeration is directly proportional to the risk of contamination with the pathogen of this disease[4,7,19]). Because the five alleles are found at the level of all three codons of interest for scrapie malady, the correct coding of the alleles should contain an index for each amino acid indicating the codon position. For example, for the ARR allele, the coding would be: A_{136}, R_{154} and R_{171}.

Several genetic variants associated with the same level of resistance / susceptibility to scrapie were found at codons 136, 154 and 171, at whose level the amino acid variations take place and which generate the respetive mutations, the wild type being transformed into the mutant type. The most common amino acids for these three codons are alanine (A) versus valine (V) to codon 136, arginine (R) versus histidine (H) to codon 154 and glutamine (Q) versus arginine (R) to codon 171 (in the most cases); Seldom, at codon 171, glutamine (Q) can be substituted by histidine (H), too. In addition to variations in the amino acids within the three codons of interest for classical scrapie, at codon 141, incriminated in atypical scrapie, leucine of the wild type is replaced by phenylalanine in the mutant type [5] (Table 1).

Phenotypic identification. Based on the melting curves obtained with the kits "LightCycler FastStart DNA Hybridization Probe MasterPLUS" and "LightCycler Scrapie Susceptibility Mutation" together with the specific PCR primers the SNP and mutation detection was possible (Table 2), by means of hybridization probes contained in the kits designed at the codon level 136, 154 and 171 of the prion protein.

Identification of genetic variants was performed by the analysis of melting curves generated by the amplification products, respectively depending on melting temperature of the complementary probes with the mutagenic site in comparison with the kit standards[14] (Fig. 1).

Table 1

Allelic variants (wild type and mutant) in codons 136, 154, 171 and 141 of the PrP gene in sheep

Codon number	Wild type		Mutant type	
	Specific codon	Amino acid	Specific codon	Amino acid
136	G<u>C</u>C	A	G<u>T</u>C	V
154	C<u>G</u>T	R	C<u>A</u>T	H
171[A]	C<u>A</u>G	Q	C<u>G</u>G	R
171[B]	C<u>A</u>G	Q	C<u>A</u>T	H
141	C<u>**T**</u>T	**L**	T<u>**T**</u>T	**F**

Table 2

Amino acids variations at the codon level 136, 154 şi 171.

Codon number	Amino acid	Genotype	Temperature °C	LightCycler program chanel
136	A	Wild type	62,3	F2
	V	Mutant type	67	F2
154	R	Wild type	56,1	F2
	H	Mutant type	51,3	F2
171	Q	Wild type	60,2	F3
	H	Mutant type	55,6	F3
	R	Mutant type	65,3	F3

Thus, depending on the localization of the signal (peak), the genotype is constructed for each sample separately at the level of the three codons subjected to analysis. The peak characteristics (number, position and shape) customize the prion genotypes. As a general rule, the homozygotes are characterized by a single peak and two peaks are characteristic for heterozygotes. For example, to obtain a peak at 65°C at the channel F3 level there is indicated the presence of resistant variant to scrapie ARR/ARR (Fig. 2). The variant with middle susceptibility to scrapie ARQ/ARQ is detected if an amplification peak was obtained at 60°C, at 705nm wavelength (the channel F3 of Light Cycler program) (Fig. 3). The heterozygote variant ARR/ARH (with a low contaminating risk with scrapie) is detected if two peaks are obtained by amplification, one at 55°C and one at 65°C at 705 nm wavelength (Fig. 4).

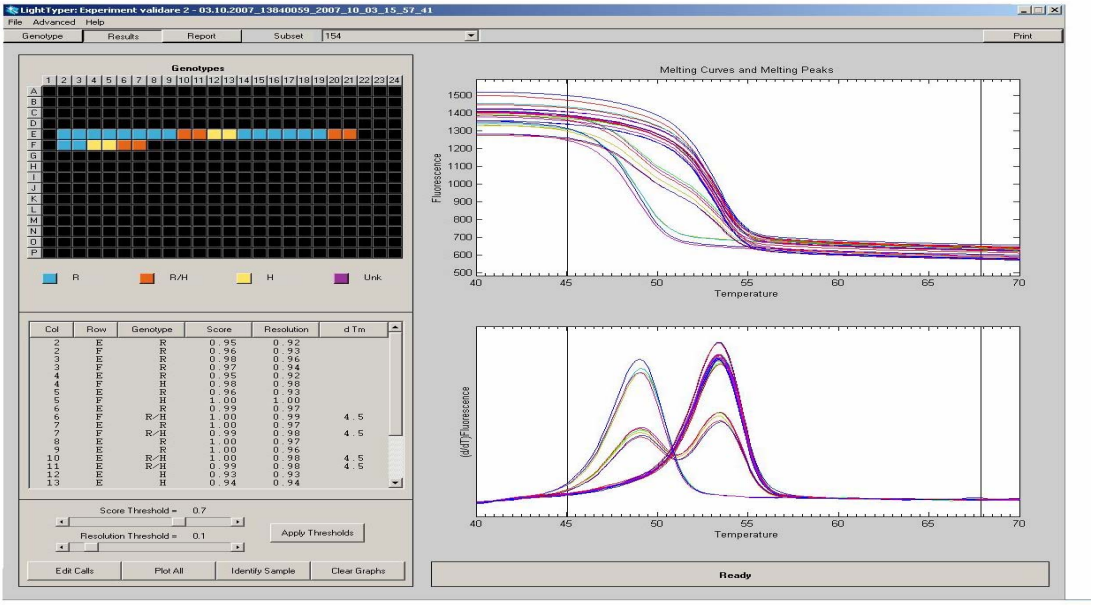


Figure 1. Melting curve analysis for phenotype identification.

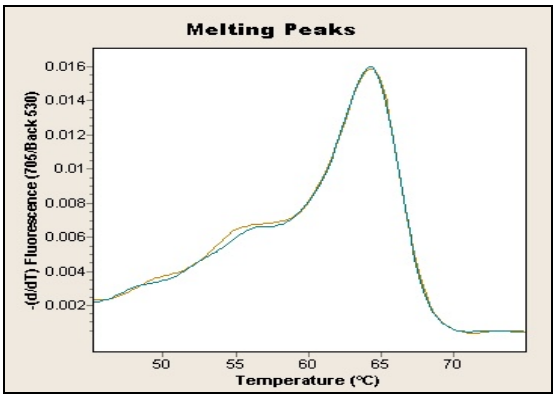


Figure 2. Melting curve for ARR/ARR genetic variant.

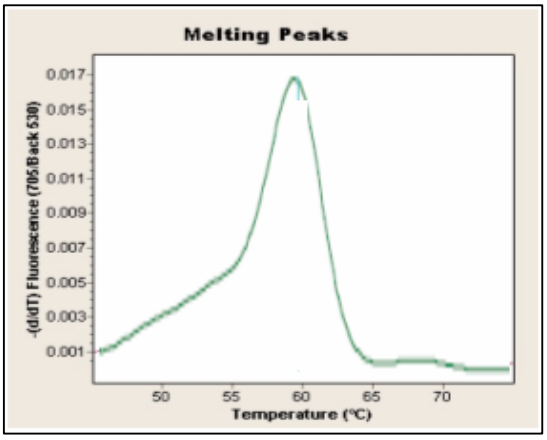


Figure 3. Melting curve for ARQ/ARQ genetic variant

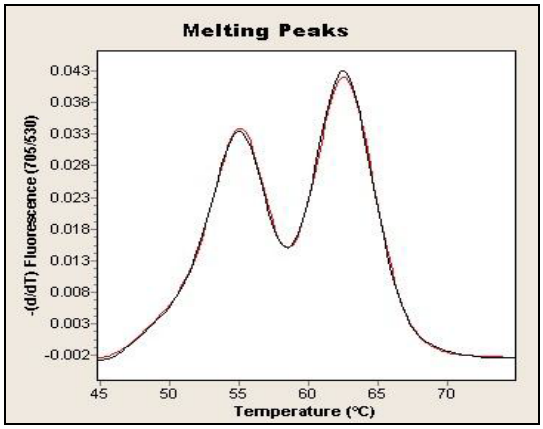


Figure 4. Melting curve for ARR/ARH genetic variant

By sequencing technique, the alignment between the PrP gene sequence prevailing in the GenBank database and the sequences derived from samples analyzed with application Clustal W of BioEdit program indicates whether there is or not there is polymorphism at codon level in the DNA fragment; the result obtained by Real-Time PCR method was confirmed also by the sequencing technique.

In the case of this sample (849458), the homology with the sequence of GenBank was 100%, any polymorphism was not identified, neither at above-mentioned three codons, or at another codon (Fig. 5).

In the case of this sample (174712), the homology with the sequence of GenBank was 99%. At codon 171 the polymorphism GAC-CGG was identified coding for amino acid arginine (Fig. 6).

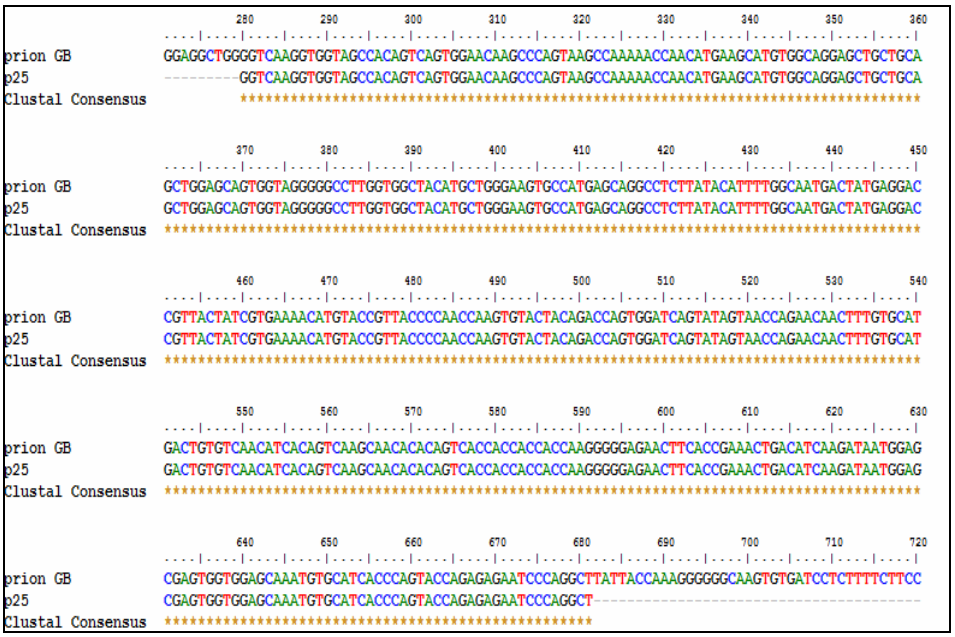


Figure 5. BioEdit alignment between the PrP gene sequence and the sequence obtained for sample 849458.

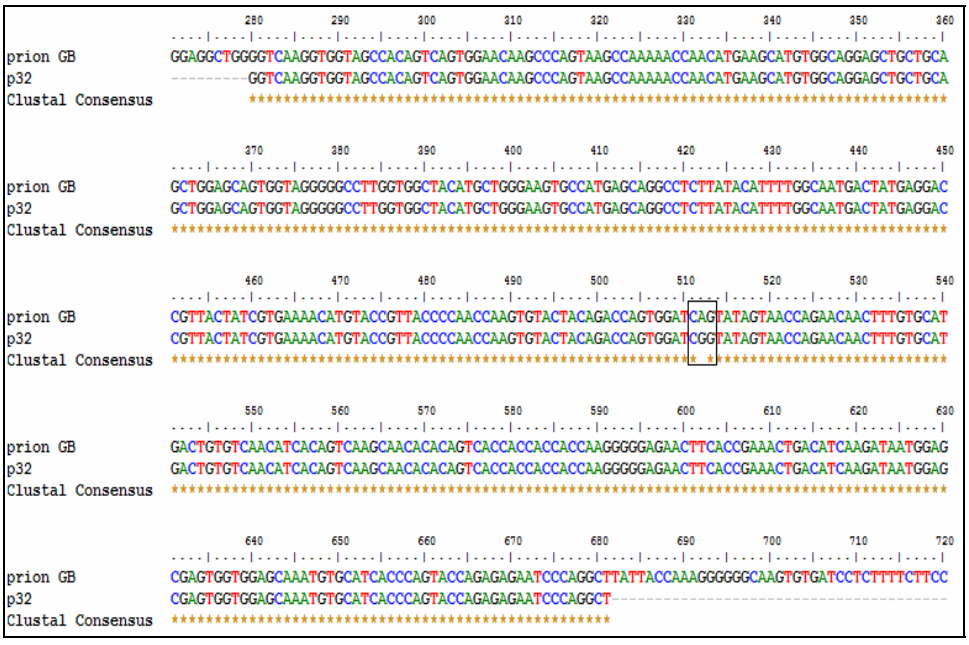


Figure 5. BioEdit alignment between the PrP gene sequence and the sequence obtained for sample 174712.

Allelic incidence. In sheep the PrP gene is located on chromosome 13[6] and has two short exons (52 bp, respectively 98 bp), containing the 5` untranslated region, and a third long exon of 4028 bp, the last exon presenting an ORF region (*open reading frame*)[10, 11]. In sheep and goats, the prion gene presents the differential processing phenomenon (alternative polyadenylation) of the mRNA[12]. The mRNA for PrP gene in sheep is detected in the brain and peripheral tissues commensing on the 98th day of gestation. The expression level remains constant from birth[12]. The multiple allelism from the PrP gene locus determines the expression of a more or less pronounced polymorphism of this protein depending on sheep breed, variety, ecotype, production type, selection and breeding systems etc.

In the Botosani Karakul sheep only three PrP alleles were found, while in the Palas Meat Breed all five alleles involved in scrapie contamination are encountered. In both sheep breeds the PrP alleles are uniformly distributed.

In the Botosani Karakul breed the ARQ allele is very common, recording a high incidence (55.39%). Also, the presence of the ARR allele is significant (40.25%). The lowest frequency is recorded by the ARH allele (4.36%). The AHQ and VRQ alleles are missing from the prionic panel of this breed (Fig. 6).

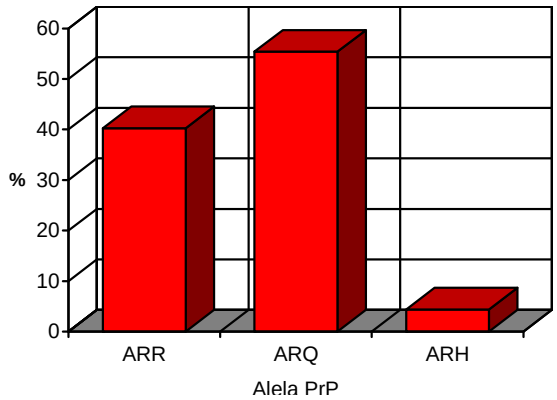


Figure 6. Allelic frequencies at the PrP gene locus in the Botosani Karakul breed.

In the Palas Meat Breed the ARR allele is the most frequent, having a rather high value (58.67%). The ARQ allele meets in a quarter of the population individuals (25.71%). Presence of the VRQ allele surprises by the significant frequency recorded (9.15%), given its impact concerning the association with spongiform encephalopathy. The ARH and AHQ alleles have low incidences (3% - 4%) (Fig. 7).

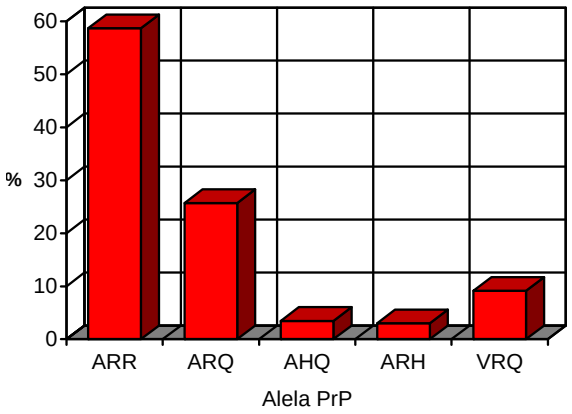


Figure 7. Allelic frequencies at the PrP gene locus in the Palas Meat Breed.

Genotypic distributions. Theoretically, through the combination of the five PrP alleles, there is the possibility of expressing the 15 prion genotypes: five homozygous (ARR/ARR, ARQ/ARQ, AHQ/AHQ, ARH/ARH, VRQ/VRQ) and ten heterozygous (ARR/ARQ, ARR/AHQ, ARR/ARH, ARR/VRQ, ARQ/AHQ, ARQ/ARH, ARQ/VRQ, AHQ/VRH, AHQ/VRQ, ARH/VRQ). This makes the PrP locus to be a polymorphic enough locus.

In the Botosani Karakul breed, the three alleles are found in six genotypic combinations: three homozygous (ARR/ARR, ARQ/ARQ and ARH/ARH) and three heterozygous (ARR/ARQ, ARR/ARH and ARQ/ARH). In the Palas Meat Breed the presence of all five alleles makes possible the expression of 14 genotypes from those 15 possible, only the genotype homozygous ARH/ARH missing from the prionic panel. The common characteristic of the two breeds is the very disproportionate spreading of the PrP genotypes. But outside the numerical expression of genotypes, the breeds are differentiated between them concerning the distributions of these genotypes (Table 3).

In the Botosani Karakul breed the homozygous individuals ARQ/ARQ are quite present in the prionic table (44.11%). An appreciable frequency is recorded also for the ARR/ARR homozygotes (27.18). The other homozygote expressed (ARH/ARH) has a sporadic spreading (0.51%). Among heterozygotes only the ARR/ARQ individuals have a notable incidence (20.51%) being the third genotype in the genotypic configuration. For the other two heterozygous genotypes the frequency is low (ARR/ARH - 5.64%) or very low (ARQ/ARH - 2.05%). The occurrence rate of homozygous individuals ARR/ARR and ARQ/ARQ is about 10% and 15% higher compared to the Hardy-Weinberg law expectations. By contrast, the ARR/ARQ heterozygotes are met in population over two times less than they were expected. At the level of the other three genotypes the differences between empirical and theoretical frequencies are not so obvious as in the previous cases. As a result of these issues, the value of χ^2 test is very high (29,767***), so that the Botosani Karakul breed is in very significant genetic disequilibrium at the PrP locus.

In the Palas Meat Breed the most frequent individuals are those possessing ARR/ARR

homozigous and ARR/ARQ heterozygous genotypes, both groups recording almost equal incidences (30.91% and 33.75%). Among homozygotes the ARQ/ARQ individuals can also be mentioned, but with a relatively low frequency (6.62%). The VRQ/VRQ homozygotes (0.63%) and especially the AHQ/AHQ ones (0.32%) are very rare in population. Among heterozygotes the ARR/VRQ individuals are evidenced by their moderate incidence (14.51%). The ARR/AHQ and ARR/ARH individuals recorded low frequencies (3% - 4%), and those of ARQ/AHQ, ARQ/ARH and ARQ/VRQ type are even uncommon (between 1% and 2%). The other heterozygotes are very rare (0.63% - AHQ/ARH and AHQ/VRQ) or sporadic in population (0.32% - ARH/VRQ). In all cases the observed frequencies of PrP genotypes comply to the Hardy-Weinberg law, the χ^2 test value (5.7623) being unsignificant.

In the Botosani Karakul breed the total homozygosis (71.80%) is 2.5 times more frequent than the total heterozygosis (28.20). But the statistical calculus shows that the homozygotes should be more frequent than heterozygotes and that the two categories of genotypes should occur in more balanced proportions (47.07%/52.93%). For this reason, the test χ^2 records a high value (24.5472***), so that the differences between the observed and expected frequencies are very significant (Fig. 8). In the Palas Meat Breed the heterozygotes (61.52%) are by over 20% more frequent than homozygotes (38.48%). However, the breed is in Hardy-Weinberg genetic equilibrium, since the differences between the observed and expected frequencies are insignificant ($\chi^2 = 0.5318$) (Fig. 9).

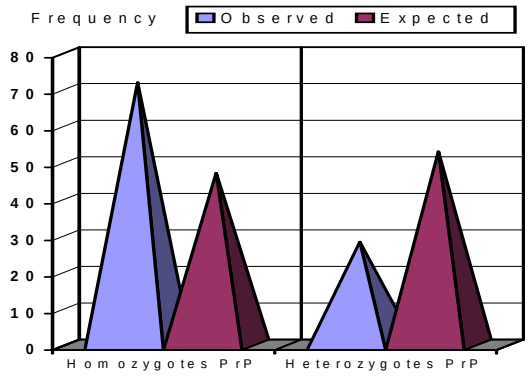
Genotyping of the Botosani Karakul sheep and Palas Meat Breed for the prion protein shows that the major differences have been reported between the two breeds concerning the genetic structure at the PrP locus related to the number of alleles and genotypes expressed and their share in the prionic panel.

Table 4

Genotypic distributions at the PrP locus in the Botosani Karakul sheep and Palas Meat Breed

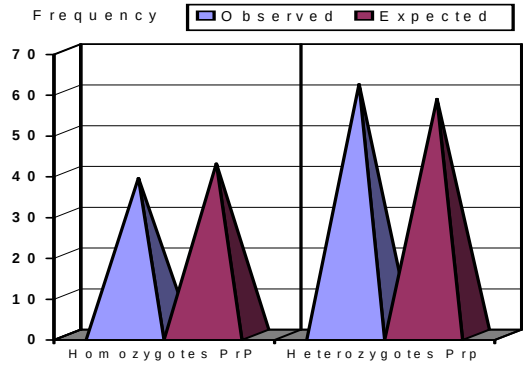
Genotype PrP	Botosani Karakul Breed			Palas Meat Breed		
	no.	observed frequency	expected frequency	no.	observed frequency	expected frequency
ARR/ARR	53	27,18	16,21	98	30,91	34,42
ARQ/ARQ	86	44,11	30,67	21	6,62	6,61
AHQ/AHQ	-			1	0.32	0.12
ARH/ARH	1	0,51	0,19	-	-	0.09
VRQ/VRQ	-			2	0.63	0,84
ARR/ARQ	40	20,51	44,59	107	33,75	30.17
ARR/AHQ				12	3,79	4,07
ARR/ARH	11	5,64	3,51	11	3,47	3,52
ARR/VRQ				46	14,51	10,74
ARQ/AHQ				4	1,26	1,78
ARQ/ARH	4	2,05	4,83	5	1,58	1,54
ARQ/VRQ				5	1.58	4,70
AHQ/ARH				2	0,63	0.21
AHQ/VRQ				2	0,63	0,64
ARH/VRQ				1	0.32	0.55
Total	**195**	**100.00**	**100.00**	**317**	**100.00**	**100.00**

$\chi^2 = 29.767$***; L.D. = 5; $p<0.001$; / $\chi^2 = 5.7623$; L.D.=14; $p>0.05$



$\chi^2 = 24.5472$***; L.D. =1; p<0.001

Figure 8. Zygosity status at the PrP gene locus in the Botosani Karakul breed

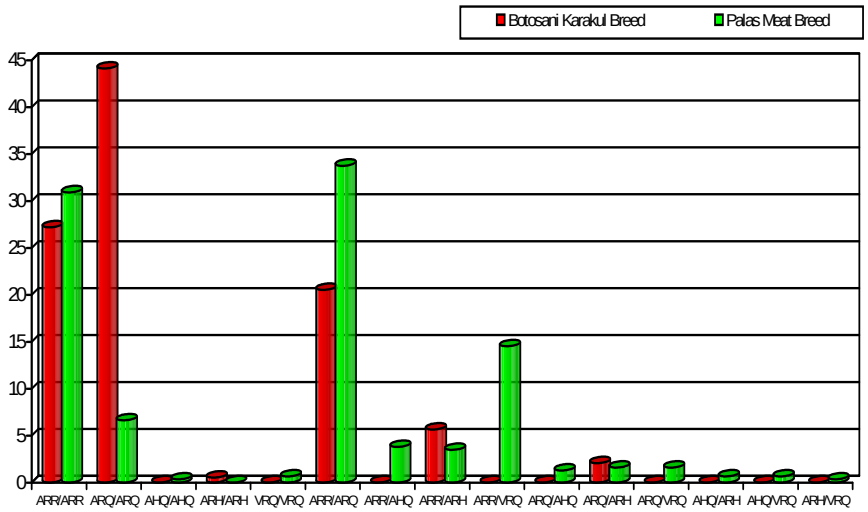


$\chi^2 = 0.5318$; L.D. =1; p>0.05

Figure 9. Zygosity status at the PrP gene locus in the Palas Meat Breed

This situation is statistically demonstrated by comparing the two empirical distributions of the prion genotypes, the differences between the two breeds being very significant, evidenced by the value of χ^2 test, both as individual genotypic distributions and as regards the overall status of the prionic zygosity (490.78***, respectively 485.97***) (Fig. 10, 11).

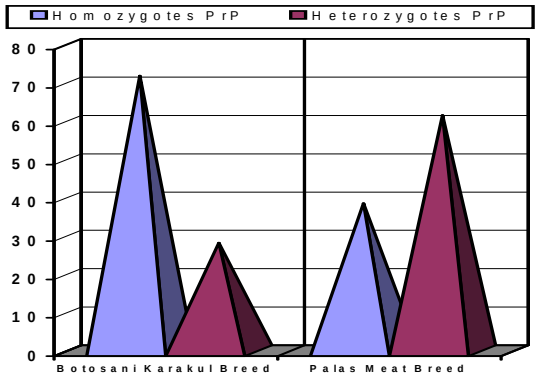
This analysis demonstrates that the Palas Meat Breed is more polymorphic than the Botosani Karakul breed at this locus. These differences could be due to the specificity of the selection criteria applied for each breed but also to breed age. In general, the Karakul sheep is the oldest ovine breed, within which a very rigorous selection was practiced to obtain lamb pelts with valuable qualitative features. Instead, the Palas Meat Breed is a new biological creation (about three decades) specialized for meat production, for which purpose also the industrial crossbreeding type was used. According to our results and by linking data from the literature[7, 8, 14, 19], one can find that as the breed is older, the polymorphism is narrower; the polymorphism is increasing along with ascending on the phylogenetic scale. On the other hand, the strong pressure of selection reduces the prion polymorphism and conversely a lessening of selection intensity leads to increasing the polymorphism of this locus. Also, if the selection is practiced with a lower exigency, containing many traditionalism elements, contribute to emphasizing the polymorphism. However, the



Genotype PrP

$\chi^2 = 490.78$***; L.D. = 14; p<0.001

Figure 10. Empiric distributions at the PrP gene locus in the Botosani Karakul sheep and Palas Meat Breed.



$\chi^2 = 485.97$***; L.D. = 1; p<0.001

Figure 11. Zygosity status at the PrP gene locus in the Botosani Karakul sheep and Palas Meat Breed

prionic polymorphism is more pronounced in hybrid entities (crossbreds). But by the polymorphism widening, the reception probability of disease also increases. But the association of PrP genotypes with the risk levels regarding the resistance / susceptibility to scrapie disease in these two sheep breeds will be the topic of another study.

CONCLUSIONS

In sheep belonging to the Botosani Karakul and Palas Meat breeds there were analyzed the existing possibilities of polymorphism at the PrP gene level (associated with susceptibility to scrapie) coding for prion protein at codons 136, 154 and 171; the investigation was performed by the Real-Time PCR technique for identifying the genotypes at this locus.

Essential differences were found between the two sheep breeds both in terms of number of PrP alleles and genotypes and their repartition within the prion array; these differences have a very significant statistical assurance.

The prionic panel configuration shows a more limited polymorphism in the Botosani Karakul breed, while in the Palas Meat Breed this polymorphism is very emphasized.

The distributional characteristics of the prionic genotypes determine the observed frequencies be in accordance with the Hardy-Weinberg expectation law in the Palas Meat Breed, while the Botoşani Karakul breed is in very significant genetic disequilibrium at the PrP locus.

The causes of differentiated polymorphism at PrP locus would be due to different selection criteria used to improve these two breeds, to selection pressure for achieving the production metabolism specificity of each breed and to genetic history of these two breeds.

The differences in the genetic structure at the PrP locus create differentiated prerequisites regarding the resistance / susceptibility to scrapie of sheep belonging to the two breeds.

REFERENCES

1. Arsac, J.N.; Andreoletti O.; Bilheude J.M.; Lacroux C.; Benestad S.L.; Baron T., *Similar biochemical signatures and prion protein genotypes in atypical scrapie and Nor98 cases, France and Norway. Emerg. Infect. Dis.,* **2007**, *13*, 58–65.
2. Benestad, S.L.; Sarradin P.; Thu B.; Schönheit J.; Tranulis M.A.; Bratberg B., *Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. Vet. Rec.* **2003.** *153*, 202–208.
3. Benestad S.L.; Arsac V; Goldmann W.; Noremark M., *Atypical/Nor98 scrapie: Properties of the agent, genetics, and epidemiology. Vet. Res.*, 2008, *39*, 19.
4. Belt P.B.; Muileman I.H.; Schreuder B.E.; Bos-Ruijter J.; Gielkens A.L.; Smits M.A., *Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. J. Gen. Virol.*, **1995**,*76*, 509-517.
5. Beringue V.; Andreoletti O.,. *Classical and atypical TSE in small ruminants*. *Animal Frontiers*, **2014**, *4 (1)*, 33-43.
6. Castiglioni B.; Comincini S.; Drisaldi B.; Motta T.; Marchitelli C.; Valentini A.; Pagnacco G.; Leone P.; Ferretti, L., *Physical mapping of the prion genes in cattle, sheep and man by fluorescence in situ hybridization. Proceed. of the 6th World Cong. on Genetics Appl. to Livestock Prod.,* **1998**, *27*, 343–346.
7. Coşier Viorica,. *Creşterea rezistenţei la scrapie la populaţiile de ovine din România prin selecţia asistată la nivel molecular*. Edit Risoprint, Cluj-Napoca, **2008**.
8. Coşier Viorica; Vlaic A.; Mireşan Vioara; Constantinescu R., *The genetic resistance of rams from Turcana breed to Ovine Transmissible Spongiform Encephalopathy (scrapie). Romanian Biotechn. Lett,* **2011**, *16, (4),* 6328-6335.
9. Fediaevsky A.; Tongue Sue C.; Nöremark Maria; Calavas D.; Ru G.; Hopp P., *A descriptive study of the prevalence of atypical and classical scrapie in sheep in 20 European countries*. BMC Vet. Res., *4 (19*), **2008,** 1746-6148.
10. Goldmann W.; Hunter N.; Foster J.D.; Salbaum J.M.; Beyreuther K.; Hope J., *Two alleles of a neural protein gene linked to scrapie in sheep. Proc. Natl. Acad. Sci. USA,* **1990**, *87*, 2476-2480.
11. Goldmann W., *PrP gene and its association with spongiform encephalopathies. Brit. Med. Bull.,* **1993**, *49*, 839-859.
12. Goldmann W.; O 'Neill G.; Cheung F.Ş.; Charleson F.; Ford P.; Hunter N., *PrP (prion) gene expression in sheep may be modulated by alternative polyadenylation of its messenger RNA. J. Gen. Virol.,* **1999**, *80*, 2275-2283.
13. Hrincă Gh.; Georgescu S.E.; Vicovan G.; Nechifor I., *Genetic and pathological aspects of prion protein (PrP) in sheep belonging to Botosani Karakul breed. AgroLife Sci. J., Sci. Pap. Univ. of Agr. Sci. and Vet. Med. Bucharest***, 2014**, 3 (1), 69-74.

14. Hrincă Gh.; Georgescu S.E.; Vicovan G.; Nechifor I., *Genetic structure at the prion protein locus (PrP) of Botosani Karakul sheep populations in relation to the accuracy and intensity of selection mechanisms. Sci. Pap., Univ. of Agr. Sci. And Vet. Med. Iaşi, Anim. Sci. Series*:, **2014,** 62, 13-21.
15. Hunter N., *Transmissible spongiform encephalopaties*. In: "Breeding for Disease Resistance in Flock Animals", 2nd Ed. International, Wallingford, 1999, pp. 325-339.
16. Kevorkian Steliana Elvira Maria; Zăuleț Mihaela; Manea Maria Adina; Georgescu S.E.; Costache Marieta, - *Analysis of the ORF region of the prion protein gene in the Botosani Karakul sheep breed from Romania. Turk. J. Vet. Anim. Sci.*, **2011**, *35(2),* 105-109.
17. Laplanche J.L.; Chatelain J.; Westaway D.; Thomas S.; Dussaucy M.; Brugerepicoux J.; Launay J.M., *PrP polymorphisms associated with natural scrapie discovered by denaturing gradient gel electrophoresis. Genomics,* **1993**, 15, 30-37.
18. Oidtmann B.; Simon D.; Holtkamp N.; Hoffmann R.; Baier M., *Identification of cDNAs from Japanese pufferfish (Fugu rubripes) and Atlantic salmon (Salmo salar) coding for homologues to tetrapod prion proteins. FEBS Lett*., **2003**, *538*, 96-100.
19. Otelea M.R.; Zaulet M.; Dudu A.; Otelea F.; Baratareanu S.; Danes D.; *The scrapie genetic susceptibility of some sheep breeds in southeast Romanian area and genotype profiles of sheep scrapie infected. Romanian Biotechn. Lett.,* **2011**, *16 (4)*, 6419-6429.
20. *Petit F.; Boucraut-Baralon C.,* From slow virus to prion: molecular biology of transmissible neurodegenerative diseases. Rev. Méd. Vét*., **1992**,* 143 (7), *595-605.*
21. Prusiner S.B., *Novel proteinaceous infectious particles cause scrapie. Science,* **1982**, *216 (4542)*, 136–144.
22. Prusiner S.B., *Molecular biology of prion diseases.* Science**, 1991**, *252 (5012)*, 1515–1522.
23. Prusiner S.B., *Prions. Proceed. of the Nat. Acad. of Sci. of USA,* 1**998**, *95 (23)*, 13363–13383.
24. Rivera-Milla E.; Stuermer C.A.O.; Malaga-Trillo E., *An evolutionary basis for scrapie disease: identification of a fish prion mRNA. Trends Gen.,* **2003**, *19*, 72-75.
25. Roche Diagnostics, PCR Manual, 2nd Edition, **1999**, 52-58.
26. Selvin P.R., *The renaissance of fluorescence resonance energy transfer. Nat. Struct. Biol*., **2000**, *7*, 730.
27. Sanger, F.; Coulson A.R.; Hong G.F.; Hill D.F.; Petersen G.B., *Nucleotide sequence of bacteriophage λ DNA*, *J. Mol. Biol.,* **1982**, *162 (4*), 729–773.
28. Simonic T.; Duga S.; Strumbo B.; Asselta R.; Ceciliani F.; Ronchi S., *cDNA cloning of turtle prion protein. FEBS Lett*., **2000,** *469*, 33-38.
29. Somerville R.A., *TSE agent strains and PrP: reconciling structure and function. Trends in Biochem. Sci.,* **2002**. *27 (12)*, 606–612.
30. Strumbo B.; Ronchi S.; Bolis L.C.; Simonic T., *Molecular cloning of the cDNA coding for Xenopus laevis prion protein. FEBS Lett.,* **2001**, *508*, 170-174.
31. Wopfner F.; Weidenhofer G.; Schneider R.; von Brunn A.; Gilch S.; Schwarz T.F.; Werner T.; Schatzl M., *Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. J. Mol. Biol.,* **1999**, *289*, 1163-1178.