GENETIC STRUCTURE AT THE PRION PROTEIN LOCUS (*PrP*) OF BOTOSANI KARAKUL SHEEP POPULATIONS IN RELATION TO THE ACCURACY AND INTENSITY OF SELECTION MECHANISMS

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Abstract

The paper investigates the fixing degree of alleles at the PrP locus in the Botosani Karakul sheep bred in two farm types: elite farm (with very rigorous selection criteria) and production farms (with lower selection exigencies). Sheep genotyping at PrP locus was performed by the Real-Time PCR technique. Significant differences were noted between the two farm types concerning the genetic structure at this locus. In sheep belonging to private farms, all five prion alleles are found: ARR, AHQ, ARH, ARQ and VRQ. In sheep of elite farm, four alleles are present, the allele VRQ lacking. This situation leads to a more emphasized polymorphism at the PrP locus in sheep of private farms revealed by expressing of 11 prion genotypes in comparison with the seven ones found in sheep of elite farm. In the elite farm, the ARR allele (associated with increased resistance to scrapie) is more frequent, causing higher incidences of genotypes containing this allele than in the private farms. Although the VRQ allele (associated with high susceptibility to develop the prion disease) is sporadically spread in private farms, it contributes to expressing of two genotypes. The ARQ allele (with a middle association level regarding the resistance / susceptibility to scrapie) is frequently found in both farm types (especially in homozygous status), but it is more common in the production farms. The elite farm is in genetic disequilibrium PrP, and the production farms conform to the Hardy-Weinberg law at this locus.

Key words: scrapie, prion, Real-Time PCR, sheep, Karakul

INTRODUCTION

Prions are infectious particles that cause a group of neurodegenerative diseases characterized by degradation of the central nervous system. These were reported in species of farm and wild animals, as well as in humans (2). In sheep the prions represent the pathogenic agent that causes scrapie (5).

The increased interest towards these disorders is due to specific peculiarities relating to the structure, transmission mode and pathology induced intra- or interspecifically by prion. All of these diseases are associated with conformational alteration determined by conversion of the normal prion protein, sensitive to protease (PrP^{C}), to a resistant form to protease (PrP^{Sc}), after the prion protein has been anchored at the cell membrane. Thus, the prion protein is a marker specific to transmissible spongiform encephalopathies (1).

Obviously, for the human food safety, the most important issue is the quality of the meat ingested as regards the risk degree of contamination with prion particles. The molecular knowledge of TSEs on its infectivity from animals to human on the basis of the properties of prion agents aforesaid mentioned, is an imperative requirement of the modern Community or Global animal husbandry (4). This all the more so since the genetics offers to improvers multiple opportunities for creating new breeds, lines or hybrids of domestic animals through different crossing (especially the industrial ones), methods which creates prerequisites for widening the genetic polymorphism of proteins, including the prion one. On the other

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The manuscript was received: 15.09.2014
Accepted for publication: 28.10.2014

hand, within a more traditional animal breeding, random or less controlled crossbreedings can take place, having the same effect. But with the proteinaceous polymorphism widening, certain genetic variants may occur which are associated with various diseases, including the scrapie.

The incidence of scrapie in sheep is a resultant of natural selection action, but may also be a reflex of artificial selection depending on the improvement technologies used within this species to strengthen the production traits. The present paper investigates the fixing mode of alleles at the PrP locus in the Botosani Karakul breed under differentiated conditions concerning the selection pressure, its intensity, as well as the breeding systems of diverse sheep populations. Having an image of this kind, there were created opportunities for reducing the risk of contamination with scrapie taking coherent measures in the improvement and reproduction process of sheep.

MATERIAL AND METHOD

Experimental works were carried out on sheep belonging to the Botosani Karakul breed. These constituted two distinct populations depending on the selection criteria applied within them: a population of 178 individuals from the elite farm of the *Research and Development Station for Sheep and Goat Breeding, Popauti-Botosani* and another composed of 2822 individuals from several private farms that are affiliated to the *Association of Sheep and Goat Breeders "MOLDOOVIS" Botosani*. The differentiation between the two types of farms concerns the accuracy measure of selection and reproduction process and the intensity of selection pressure, attributes that are most pronounced in the elite farm in comparison with the breeding (production) farms.

In order to identify the prion protein there were used blood samples and brain fragments from which the DNA was extracted and purified by special laboratory techniques. Identifying works of genetic variants of protein prion in sheep were performed following the instructions of the PCR Manual edited by Roche Diagnostics (12).

The *blood samples* were taken from the jugular vein into vacutainers containing EDTA as an anticoagulant. The blood samples were stored at 4°C and processed as quickly as possible.

The brain fragments were collected from brainstem by conventional surgical methods, performing the following sequences (figure 1):
1 - the dorsal side of head is placed on the surface of work table so that the occipital hole to be directed to the operator.
2 - with special attention the bulb and spinal cervical (which protrudes from the occipital hole) are separated of *duramater* by cutting cranial nerves with scissors. Through these operations the caudal brainstem which becomes free in *foramen magnum* will be able to view.
3 – a spatula is inserted between the bony floor of the occipital hole and the free part of the brainstem; the spatula is directed cranially and cranio-ventrally over a distance of about 4-7 cm long; then, by some laterality movements, the brainstem is sectioned 2-3 cm cranially beyond the "obex".
4 – lastly, the spatula retracts easily together with the brainstem fragment collected.

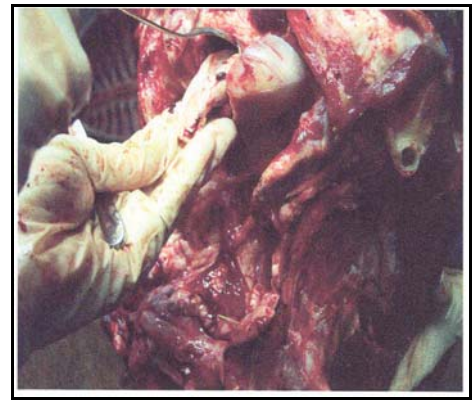


Figure 1 Sampling the brainstem for analyzing the prion protein.

DNA extraction from blood using the kit "Genomic Wizard" (Promega). Isolation of genomic DNA is performed by a saline extraction process in four stages. In the first phase the red cell lysis occurs followed by white cell lysis and of their nuclei. In the next step the proteins are precipitated with a concentrated salt solution while the genomic

DNA with high molecular weight remains in solution. Finally, the genomic DNA is concentrated and desalinated by precipitation with isopropanol.

DNA extraction from brain homogenate using the kit "High Pure PCR Template Preparation" (Roche). Isolation of genomic DNA is performed using the kit "High Pure PCR Template Preparation" from Roche in four stages. In the first phase the cells are lysed as a result of their incubation with proteinase K and chaotropic salts (guanidine hydrochloride). Following this treatment, the destruction of cellular architecture occurs, the effect being the release of components in solution concomitantly with nuclease inactivation. The nucleic acids are selectively bound at the level of spin columns and subsequently washed in the two phases to remove all contaminating cellular components. In the last phase the elution solution, that is poor in salts, releases nucleic acids from the spin column level. This method eliminates the use of organic extraction and precipitation solvents, which allows a rapid purification of multiple samples simultaneously.

Genotyping of the animals at the PrP locus was performed by the Real-Time PCR technique which is based on the fluorimetric detection principle in real time using the detection kits LightCycler Scrapie Susceptibility Mutation Kit (TIB MOLBIOL) and LightCycler FastStart DNA Master Hybridization Probes (Roche). These kits contain hybridization probes designed at the level of codons 136, 154 and 171 of the prion protein, allowing the fluorimetric detection and evaluation of the PCR products by means of fluorochromes which are coupled to oligonucleotidic hybridization probes with specific sequences "Hybridisation probes" and which detects only specific PCR products within the FRET phenomenon (13). (figure 2). The genotype determination is possible by melting curve analysis after the amplicon formation. Thus, one of the probes (anchor probe) hybridizes to a gene region with low mutagenic potential. The sequence of the second probe is complementary to the mutagenic site. Also, the anchor probe benefits from a higher melting temperature (*Tm*), so that the fluorescent signal emitted during the melting curve analysis is given strictly by the probe comprising the mutant site. The melting temperature (*Tm*) of the latter is influenced, in addition to Adenine and Guanine content (ratio A-T/G-C), on the degree of complementarity between this and the hybridization site: thus, in the presence of a base pair which is not complementary (single nucleotide polymorphism), *Tm* is lower.

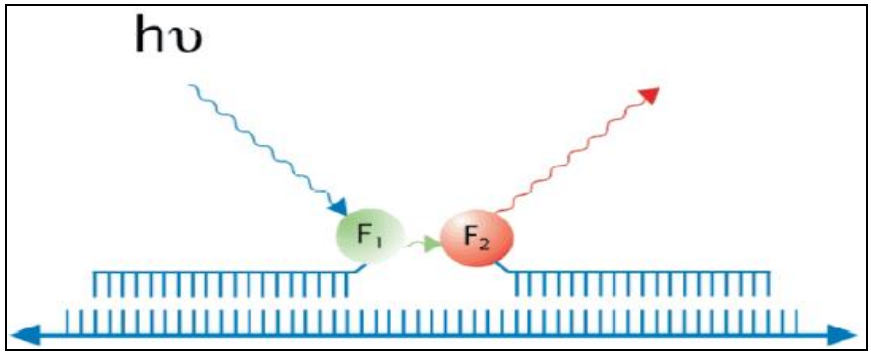


Figure 2 Representation of Fluorescence Resonance Energy Transfer (FRET) phenomenon: measurable fluorescence emission produced by the proximity of the two oligonucleotidic probes (by Salvin, 2000)

The genotyping reaction presented three phases: i) sample denaturation; ii) PCR amplification of the target fragment; iii) obtaining melting curves used later in the analysis.

In order to develop the Real Time PCR reaction the following reagent mixture was carried out (table 1):

Table 1 Components of Real Time PCR reaction

Components	Volume (µl)
$MgCl_2$	0,8
Primer mixture and probes	4
Polymerase mixture, dNTP, reaction buffer, $MgCl_2$	2
Deionised water	11,2
Total volume	**18**

The Real-Time PCR reaction was performed on Light Cycler 2.0 apparatus (Roche), its development requiring several steps (table 2).

For amplification, in the PCR reaction two oligonucleotide primer sequences were used to determine synthesize of the direct chain (sense or forward primers) and of the complementary chain (antisense or reverse primers) (table 3).

Table 2 Real Time PCR reaction scheme

Program	Cycle number	Temperature	Time	Acquisition mode
Pre-Incubation	1	95°C	8 min	none
Amplification	45	95°C	10 sec	none
		60°C	10 sec	single
		72°C	15 sec	none
Melting curve	1	95°C	2 min	none
		45°C	60 sec	none
		75°C	0 sec	continuous
Cooling	1	40°C	30 sec	none

Table 3 Primer sequences used in amplification

Locus	Primer sequence	Amplified fragment length (pb)
Gene encoding of PrP protein	Forward: GGTCAAGGTGGTAGCCACAGTCAGTGGAAC Reverse: ATCACCCAGTACCAGAGAGAATCCCAGGCT	402

Achieving the melting curve of amplification products is need to identify the alleles at the PrP locus level depending on the number, position and shape of the peaks obtained by heating the reaction medium by 0.2°C/sec starting from the temperature 45°C to 75°C.

Reconfirming the results obtained by Real Time PCR technique was performed by the sequencing technique. The primers used in the sequencing phase were designated at the level of exon 3, so as to be amplified a fragment 402 bp from position 22556 to position 22958 of the ovine prion gene (GenBank accession number U67922). This fragment contains the three polymorphic codons: 136, 154 and 171.

There were calculated the allelic and genotypic frequencies at the PrP locus and the ratio between homozygosity and heterozygosity at this locus. The Hi^2 test (χ^2) was used to see if the populations analyzed conform to the genetic equilibrium law. To highlight the differences in the genetic structure of populations analyzed the comparing method of two empirical distributions was used.

RESULTS AND DISCUSSIONS

Initially, the DNA samples extracted from blood cells and brain homogenate were quantified spectrophotometrically and electrophoretically.

At the level of PrP gene of sheep there have been described a number of 13 polymorphic codons located between codons 98 and 234 (9, 11), but the relevant codons concerning the association with resistance/susceptibility to scrapie are located in positions 136, 154 and 171 (6).

Out of the 15 alleles of the PrP gene, only five are used to characterize the sheep at the PrP locus for scrapie malady: ARR, AHQ, ARH, ARQ and VRQ, this sequence denoting the decreasing effect of the resistance to contracting the disease (3, 10).

Phenotypic expression. The identification of specific genetic variants of the prion protein for each animal tested was carried out on the basis of melting curves obtained according to the number, position and shape of peaks contained therein. The configuration of peaks is given by the nucleotide structure of the amplification products at the temperature at that the changes of PCR reaction medium occur; depending on these characteristics, the genotypes at the level of ovine prion gene are detected. An example regarding the typical phenotypic profile of the ARR/ARR genotype is shown in figure 5.

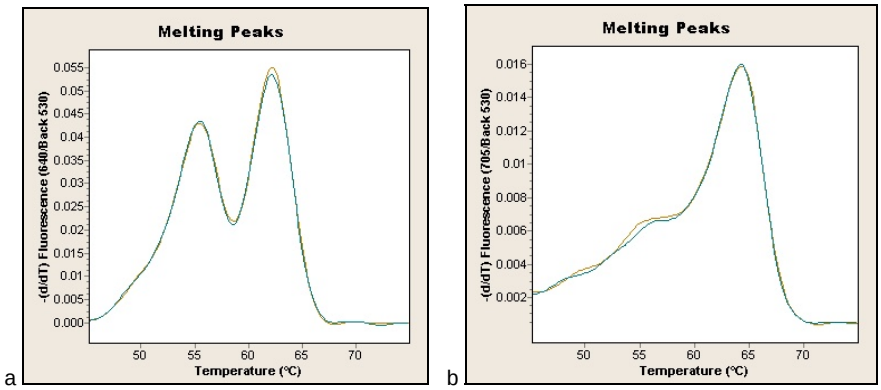


Figure 5 Real-Time PCR results obtained at codons 136 and 153 (a) and 171 (b), the genotype determined being the homozygote ARR/ARR

Because the Real-Time PCR technique is highly sensitive, the presence of contaminants may influence the melting curves used in genotyping analysis. This is applicable from the time of DNA isolation when there is the possibility that some inhibitors to be present and that may influence the Real Time PCR reaction. To confirm the results obtained by Real Time PCR technique, some of the samples were analyzed by the sequencing technique, the results being those expected (figure 6).

Figure 6 Confirming the Real-Time PCR results sequencing technique: a) lack of polymorphism; b) identifying the polymorphism

Allele spreading. The polymorphism of prion gene (associated with susceptibility to scrapie) in the Botosani Karakul sheep is due to the polyallelism met at the locus PrP.

If in sheep belonging to private farms all five prion alleles are met, in the Botosani Karakul from elite farm four alleles are found, the allele VRQ missing. In both farm types the alleles ARQ and ARR are the most widespread. But in the elite farm the ARQ allele (59.83%) is more common with about 25% than ARR allele (35.11%), whereas in the private farms the gap between the two alleles is much larger, the allele ARQ being met in nearly three quarters of herds (73.51%) and only about a quarter of the populations (23.49%) have the allele ARR. Only 3% of private farm individuals possess the other three alleles, most obvious being ARH allele (1.65%), the other two having sporadic appearances (0.66% for VRQ allele and 0.69% for AHQ allele). In the elite farm, in addition to those two alleles, the allele ARH may be mentioned although it has a

relatively low incidence (4.78%); the AHQ allele has a singular presence (0.28%), this aspect being inexplicable, since previous studies relating to this population have not revealed its presence (7, 8) (figure 7).

Genotypic structure. The existence of the five prion alleles in ovine species favours genotypically the existence of a very marked polymorphism at the PrP locus level through the following allelic combinations: 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).

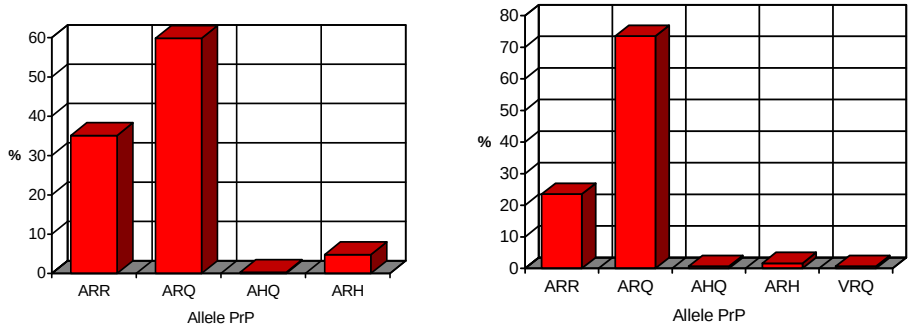


Figure 7 Allelic structure at the PrP gene locus in the Botosani Karakul sheep depending on the farm type

The differences between the two farm types are even more obvious under the aspect of the genotypic structure, both as number of genotypes expressed as well as their incidences (table 4). In the private farms, the homozygotes VRQ/VRQ and heterozygotes AHQ/ARH, AHQ/VRQ and ARH/VRQ are missing from the prionic panel. In sheep from the elite farm only seven genotypes are found: three homozygous (ARR/ARR, ARQ/ARQ and ARH/ARH) and four heterozygous (ARR/ARQ, ARR/AHQ, ARR/ARH and ARQ/ARH). In both farm types the PrP genotypes have extremely uneven distributions. In both farm types the individuals ARQ/ARQ are the most widespread, but in the private farms (55.45%) this genotype is with about 10% more frequently than in the farm elite (47.75%). In the elite farm the genotypes ARR/ARR and ARR/ARQ achieved important frequencies having a similar dissemination (21%-22%). But in the private farms the genotype ARR/ARR has a relatively low incidence (6.70%) and the genotype ARR/ARQ can be found often enough (31.96%). The individuals ARR/ARH, although they have a relatively low frequency, occupy an important position in the prionic panel of elite farm (6.18%), while in the private farms their presence is barely perceptible (0.78%). In both farm types the heterozygotes ARQ/ARH have a similar spread recording low frequencies (around 2.4%) and homozygotes ARH/ARH and heterozygotes ARR/AHQ are found sporadically in populations. For the other genotypes (homozygote AHQ/AHQ and heterozygotes ARR/VRQ, ARQ/AHQ and ARQ/VRQ), found only in the private farms, the incidences are extremely low.

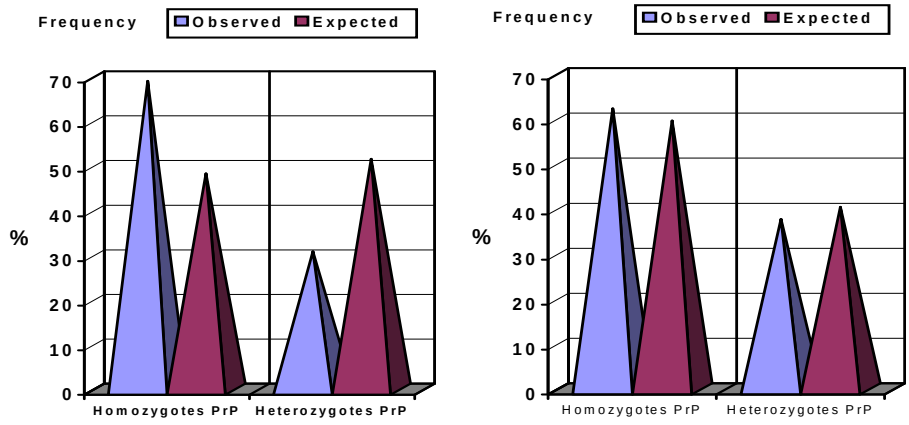
In the elite farm there are large discordances between the observed and expected frequencies, especially at the level of genotypes ARR/ARR, ARQ/ARQ and ARR/ARQ, for which reason the population is in very significant genetic disequilibrium. In the private farms the empirical distributions are similar to the theoretical distributions, so that the sheep livestock comply with the Hardy-Weinberg law (table 4).

Table 4 Genotypic structure at the PrP locus in the Botosani Karakul sheep depending on the farm type

Genotype PrP	Elite farm			Private farms		
	no.	observed frequency	expected frequency	no.	observed frequency	expected frequency
ARR/ARR	**37**	20.79	12.33	**189**	6.70	5.52
ARQ/ARQ	**85**	47.75	35.80	**1565**	55.45	54.04
AHQ/AHQ	**-**	0.00	0.00	**1**	0.04	0.01
ARH/ARH	**1**	0.56	0.23	**2**	0.08	0.03
VRQ/VRQ	**-**	0.00	0.00	**-**	0.00	0.00
ARR/ARQ	**39**	21.91	42.01	**902**	31.96	34.54
ARR/AHQ	**1**	0.56	0.20	**16**	0.57	0.32
ARR/ARH	**11**	6.18	3.35	**22**	0.78	0.78
ARR/VRQ		0.00	0.00	**8**	0.28	0.31
ARQ/AHQ		0.00	0.33	**21**	0.74	1.01
ARQ/ARH	**4**	2.25	5.72	**67**	2.37	2.42
ARQ/VRQ		0.00	0.00	**29**	1.03	0.97
AHQ/ARH		0.00	0.03		0.00	0.02
AHQ/VRQ		0.00	0.00		0.00	0.01
ARH/VRQ		0.00	0.00		0.00	0.02
Total	**178**	**100.00**	**100.00**	**2822**	**100.00**	**100.00**

χ^2 = 25.3874***; L.D.=7; p<0.001; / χ^2 = 0.9895; L.D.=13; p>0.05

In terms of the overall status of PrP zygosity in both farm types the homozygotes (between 63% and 70%) predominate over heterozygotes (between 30% and 37%), the homozygotes being by 7% more common in the elite farm than in the private farms (the situation reverses in the heterozygote case). The differences between the two farm types are not just because of their practical distributions, but rather as regards the expectation of genotype expression. In particular farms the differences between these distributions lead to a small value of the test χ^2, while in elite farm the differences between the observed frequencies and those expected determined like the population to deviate very significantly from the genetic equilibrium law (figure 8).



χ^2= 17.2243***; L.D.=1; p<0.001
Elite farm

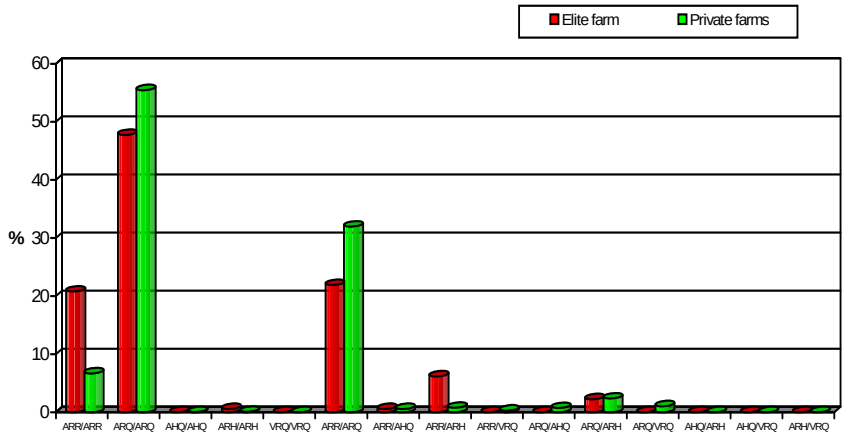
χ^2= 0.2938; L.D.=1; p>0.05
Private farms

Figure 8 Zygosity status at the PrP gene locus in the Botosani Karakul sheep depending on the farm type

Comparing the empirical frequencies of the two rows of prion genotypes it comes out that the two types of farm are distinguished very significantly both as individual genotypic distributions and as regards the overall status of the prionic zygosity, the test χ^2 recording very high values, 1153.5 *** respectively 672.36 *** (figures 9, 10).

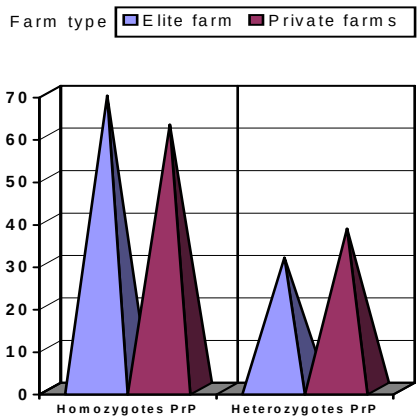
*

Analysis of the prion protein in Botosani Karakul sheep indicates that the genetic structure at the PrP locus is influenced by the selection pressure to which individuals are subjected by improvement process. Consequently, the populations of the two farm types are distinguished as regards the number of alleles found at the PrP locus, the number of genotypes expressed and their share in the prionic panel. By production and scientific specificity in the elite farm the selection pressure is stronger than in production farms. In the elite farm the selection criteria are fulfilled with the greatest strictness to improve the physical and morphological features of lamb pelt curling, strengthening the colours and colour shades of hair fibres or for creating the zootechnical lines.



χ^2= 1153.5***; L.D.=44; p<0.001

Figure 9 Empiric distributions at the PrP gene locus in the Botosani Karakul sheep from two farm types



χ^2= 672.36***; L.D.=2; p<0.001

Figure 10 Zygosity status at the PrP gene locus in the Botosani Karakul sheep from two farm types

To achieve these goals at the highest standards, different mating systems are practiced either homogeneous or heterogeneous, and not least the moderate inbreeding. As a result of the use of such quite elaborated schemes, the Karakul from elite farm is bred in pure breed. In private farms the selection is practiced with a lower exigency, containing many traditionalism elements too. In addition, the breed purity might be altered by some crossbreeding actions practiced by private shepherds (especially to increase the milk production). As such the intensity and accuracy of selective process can have an impact on fixing the alleles at the locus PrP and on their exteriorization in different genotypic combinations. From the experimental data presented, it is understood that if the selection is more pressing the prion polymorphism is narrower (in elite farm) and it becomes more pronounced as the intensity of selective process decreases. Also, the crossbreeding phenomenon (implicitly the breed impurification by gene "import") may contribute to the polymorphism widening at this locus (in private farms). In addition, the selection characteristics are reflected also on the Hardy-Weinberg law compliance character at the PrP locus. A more limited polymorphism in the farm elite determines a very significant

genetic imbalance at the PrP locus, while a more pronounced polymorphism found in production farms determines the sheep populations to be in Hardy-Weinberg equilibrium for this gene locus.

This configuration of the prion genotype array in Botosani Karakul breed may have consequences on the degree of resistance/susceptibility to scrapie of sheep bred in different exploitation systems. In the elite farm the ARR allele which is associated with high resistance to scrapie is more frequent than in the private farms. As such, the incidences of genotypes with this allele are higher in this farm type than in the private farms. The VRQ allele that is associated with high susceptibility to contract the prion disease, although it is sporadically spread in the private farms, however it contributes to expressing of the two genotypes (but only in heterozygous status). The ARQ allele, which has a middle association level on resistance / susceptibility to scrapie, is frequently found in both farm types (especially in homozygous status), but it is more common in production farms. But detailed issue concerning the prion protein polymorphism association with scrapie disease in the Botosani Karakul sheep bred in different husbandry systems will be the subject of a future paper.

CONCLUSIONS

1. Sheep of Botosani Karakul breed bred in two farm types (elite and production), distinguished between them as concerns the application of selection criteria, were genotyped at the PrP locus (associated with susceptibility to scrapie) by the Real Time PCR technique.

2 The two farm types differ very significantly regarding the genetic structure at the PrP locus: number of prion alleles and genotypes, their distributions and shares, genetic equilibrium.

3. The selection pressure influences the fixing mode of alleles at the locus PrP: more rigorous selection restrains the prion polymorphism and determines genetic disequilibrium (in elite farm) and the selection exerted with a lower accuracy leads to the polymorphism widening and to conformance with the Hardy-Weinberg law (in production farms).

4. Limiting the polymorphism, the incidence of alleles that are associated with a high resistance to scrapie increases (in elite farm), while the polymorphism emphasizing favours the expression of some alleles too that increase the susceptibility for this malady (in production farms).

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