

## THE RESPONSE OF PRION GENIC VARIATION TO SELECTION FOR SCRAPIE RESISTANCE IN HUNGARIAN INDIGENOUS SHEEP BREEDS

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The authors studied the present status of Hungarian indigenous sheep breeds based on the genetic background of scrapie resistance. The aim of this investigation was to estimate the relative frequency of prion haplotypes, genotypes and risk categories, as well as to reveal the efficiency of the scrapie eradication programme achieved over the last decade. A novel approach in the characterisation of prion by using its genic variation was also implemented. The authors established that the proportion of deleterious sites (%) can be a useful indicator of the eradication programme. Based on a large sample size, it was confirmed that the scrapie resistance of the Cikta breed is low, and the classification of this breed according to risk category has not improved. However, the frequent genotype ARQ and risk category 3 can also be considered characteristic of the breed. The careful use of these genotypes is permitted and will contribute to the maintenance of breed diversity. The response of prion genic variation to selection for scrapie resistance in the other breeds (Tsigai, Milking Tsigai, White Racka, Black Racka and Gyimes Racka) was definitely successful.

**Key words:** Genic variation, scrapie resistance, indigenous sheep breeds, breed conservation

Scrapie is an important disease of small ruminants all over the world (Dexler, 1931; Rabenau, 2009). As a generally lethal, infectious, notifiable disease caused by a prion, it is manifested in a degenerative change of the brain substance (Prusiner, 1982; Bostedt and Dedié, 1996). PrPC, which is a cellular protein, is located on the surface of neurons. PrPSc, which is the pathogenic isoform of the former, is not

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broken down at the cell membrane by the enzyme proteinase K, compared to the normal protein (PrPC), and multiplies indefinitely, thus destroying all cells (Foster and Hunter, 1998; McCutcheon et al., 2005; Kang et al., 2017).

Regulation No 999/2001 of the European Community (European Commission, 2001) lays down rules for the prevention, control and eradication of transmissible spongiform encephalopathies (TSEs) in sheep and other species.

According to Hungarian state regulations, since 2005 brain tissue samples for prion testing must be taken from slaughtered and fallen animals, and from animals culled in the framework of eradication (infected herds). Additionally, genotyping for scrapie resistance must also be carried out for the selection programmes including those of autochthonous breeds.

The first known case of scrapie in Hungary was diagnosed in 1964 (Áldásy and Süveges, 1964). The infected herds were then eradicated and Hungary remained free of scrapie for decades (Kluge and Glávits, 1993).

According to a report to Parliament by the Minister of Agriculture the incidence of the disease in its clinical form has not been observed in Hungary. In 23 cases atypical scrapie was found. For two years, intensive monitoring studies have been under way for flocks infected with atypical scrapie, thus avoiding the total depletion of flocks (Government of Hungary, 2017). The Hungarian Sheep and Goat Breeders' Association (MJKSZ) excludes VRQ carriers from breeding. Following the Hungarian national breeding programme, all candidate rams should be genotyped and only risk group R1-3 rams are allowed to be bred. Preferably, R3 rams should be avoided. Animals with the VRQ allele may leave the flock only for slaughter.

Our aims were to estimate the frequency of prion haplotypes and genotypes as well as risk categories in all Hungarian indigenous sheep breeds and to compare them to the results obtained 10 years ago in order to demonstrate the effectiveness of the prevention programme.

The works of Fésüs et al. (2004, 2008) were the first investigations of Hungarian sheep breeds in this field and served as a control condition just before the national programme (FVM, 2004) entered into force (although the Milking Tsigai was missing from that pool, and the White and Black Rackas were treated as a common stock).

## Materials and methods

### *The Hungarian indigenous sheep breeds*

Over the course of Hungarian animal husbandry several sheep breeds have been bred and have become the native breeds of the country. These multipurpose fallow breeds of low economic advantage have been receiving subsidies for preservation as biological resources since the middle of the 20th century. The following six breeds make up our heritage breeds: White Racka, Black Racka, Gyimes Racka (or Turcana, in the Romanian language Țurcană), Cikta, Tsigai and Milking Tsigai (Bodó, 2000).

### *Sampling and analysis*

Biological samples (ear cartilage tissue) were collected by employees of the Hungarian Sheep and Goat Breeders' Association during the sorting of male breeding candidates using TypiFix™ tissue sampling tags (Agrobiogen GmbH, 2016). Samples of all the registered ewes (from the year 2013 to 2015, n = 5049) were analysed to get a complete picture of the genetic susceptibility to scrapie.

The samples were sent to the Laboratory of Agrobiogen GmbH for typing the characteristic parts of the prion protein gene. Variants (haplotypes) were determined through pyrosequencing. In codon 136 alanine (A, more resistant) or valine (V) appears, while in codon 154 arginine (R, more resistant) or histidine (H) and in codon 171 arginine (R, more resistant) or glutamine (Q) and histidine (H) appear. As for the genotypes, the homozygous A<sub>136</sub>R<sub>154</sub>R<sub>171</sub>/A<sub>136</sub>R<sub>154</sub>R<sub>171</sub> genotype is the most resistant, while the homozygous V<sub>136</sub>R<sub>154</sub>Q<sub>171</sub>/V<sub>136</sub>R<sub>154</sub>Q<sub>171</sub> genotype is the most susceptible (Baylis et al., 2002).

### *Statistical analysis*

The required data were taken from the Microsoft Excel database and statistically evaluated using the Dell statistics program (Dell Inc., 2015). The relative frequency of prion haplotypes was determined. Using the Chi<sup>2</sup> test, the current condition (2013–2015) was compared to the former one (Fésüs et al., 2004, 2008).

For the analysis of genic variation the following parameters were first investigated:

effective number of haplotypes,

$$A_e = \frac{1}{\sum p_i^2},$$

Shannon's Information Index,

$$H_{Shannon} = -\sum_{i=1}^n p_i \ln p_i,$$

relative Shannon's Information Index ( $I_{rel}$  %) which is the proportion of the actual Shannon's value and the theoretically highest Shannon's value (under equal allele frequency condition).

Nei's diversity applied for haplotypes,

$$H_{Nei} = \frac{1}{k} \sum_{i=1}^n [1 - \sum p_i^2],$$

where  $k$  is the total number of haplotypes,  $p$  is the frequency of haplotypes, proportion of deleterious sites V<sub>136</sub>, H<sub>154</sub>, Q<sub>171</sub> and H<sub>171</sub> (%).

Later on the genotype frequencies were described concerning their change in time and actual genetic equilibrium (Hardy–Weinberg) status, as well as the risk group frequencies. These were all proven by the Chi<sup>2</sup> test.

## Results

Table 1A shows the distribution of the haplotypes in Cikta and Tsigai sheep populations from current years (2013–2015), where the most frequent ARQ haplotype (from 49.81 to 74.93%) was consistently followed by ARR (from 14.19 to 45.81%). The occurrence of ARH (with the exception of the Milking Tsigai, where it is 9.93%) and VRQ haplotypes is a fact which is not considered meaningful. In the Cikta breed the most resistant haplotype (ARR) shows much lower values than desirable, both in the current and in the previous analysis (2004). Concerning all haplotypes, the Chi<sup>2</sup> test does not indicate a significant difference ( $P = 0.519$ ) between the current and the previous results. It should be mentioned, however, that in the previous analysis made by Fésüs et al. (2004, 2008) on a small sample size, the haplotypes ARH and VRQ were missing.

The native Tsigai breed has undergone a remarkable improvement regarding the most preferable haplotype ARR ( $P = 0.006$ , Table 1A), and the present population of Milking Tsigai too shows higher and simultaneously more acceptable figures than the Cikta.

For the different Racka groups of the past and present, data can be found in Table 1B. As a general tendency it can be stated that there are significant changes in haplotypes; the most obvious and desirable one was found in the Gyimes Racka breed ( $P < 0.001$ ).

The genic variation is evaluated collectively based on the results presented in Tables 1A and 1B. As regards the effective haplotype number, the highest variability was found in the present White Racka population. Here, its value reached almost four out of five (3.96) and reflected the almost equilibrated haplotype frequencies of that breed. The lowest effective haplotype number could be observed in the present-day Cikta population (1.69), which is the consequence of the dominant haplotype ARQ (74.93%). Furthermore, it was found that with the increase of the favourable haplotype ARR frequencies the effective haplotype number also increased (in the breeds Tsigai and Gyimes Racka).

The values of Shannon's Information Index, which characterises the entropy of a pool, are well correlated with the effective haplotype number. However, the relative Shannon's Information Index ( $I_{rel} \%$ ) may reveal a bigger change by time than the Shannon's Information Index in the Cikta breed. This figure is advantageous when different haplotype (allele) numbers are to be compared (Gáspárdy et al., 2014). This is exemplified by the fact that despite the increase in the number of haplotypes, due to the overwhelming superiority of ARQ, entropy decreased (from 72.3 to 46.3%). The values of the relative Shannon's Information Index fell within a wider range (46.3–90.3%) in the entire sheep database examined.

Nei's haplotype diversity followed the above tendencies found in effective haplotype numbers.

**Table 1A:** Prion haplotypes (%) of Cikta and Tsigai sheep and their genic variations

Groups	Cikta (2004)	Cikta (2013–2015)	Tsigai (2004)	Tsigai (2013–2015)	Milking Tsigai (2013–2015)
Haplotypes:	(2n = 138)	(2n = 2290); $\text{Chi}^2 = 3.235$ ; df = 4; P = 0.519	(2n = 128)	(2n = 2122); $\text{Chi}^2 = 14.328$ ; df = 4; P = 0.006	(2n = 1652)
ARR	20.29 (28)	14.19 (325)	28.91 (37)	45.81 (972)	27.48 (454)
AHQ	9.42 (13)	10.70 (245)	3.13 (4)	3.30 (70)	1.27 (21)
ARH	0.00 (–)	0.13 (3)	1.56 (2)	0.42 (9)	9.93 (164)
ARQ	70.29 (97)	74.93 (1716)	65.63 (84)	49.81 (1057)	57.32 (947)
VRQ	0.00 (–)	0.05 (1)	0.78 (1)	0.66 (14)	4.00 (66)
Effective haplotype number	1.84	1.69	1.94	2.18	2.41
Shannon's index	0.794	0.745	0.846	0.874	1.087
Relative Shannon's index	72.3	46.3	52.6	54.3	67.6
Nei's haplotype diversity	0.1647	0.1459	0.1694	0.1927	0.2082
Deleterious sites, %	29.7	32.2	25.0	19.4	25.9

**Table 1B:** Prion haplotypes (%) of Racka sheep and their genic variations

Groups	Racka (2004)	White Racka (2013–2015)	Black Racka (2013–2015)	Gyimes Racka (2004)	Gyimes Racka (2013–2015)
Haplotypes:	(2n = 280)	(2n = 1688); $\text{Chi}^2 = 17.585$ ; df = 4; P = 0.001	(2n = 1558); $\text{Chi}^2 = 14.841$ ; df = 4; P = 0.005; ( $\text{Chi}^2 = 39.675$ ; df = 4; P < 0.001) <sup>+</sup>	(2n = 114)	(2n = 788); $\text{Chi}^2 = 55.814$ ; df = 4; P < 0.001
ARR	23.93 (67)	34.48 (582)	30.87 (481)	27.19 (31)	56.98 (449)
AHQ	17.86 (50)	21.92 (370)	8.22 (128)	0.88 (1)	2.92 (23)
ARH	12.14 (34)	15.34 (259)	9.11 (142)	0.00 (–)	2.03 (16)
ARQ	42.50 (119)	24.64 (416)	48.85 (761)	70.18 (80)	34.77 (274)
VRQ	3.57 (10)	3.61 (61)	2.95 (46)	1.75 (2)	3.30 (26)
Effective haplotype number	3.50	3.96	2.86	1.76	2.23
Shannon's index	1.388	1.453	1.240	0.715	0.983
Relative Shannon's index	86.3	90.3	77.1	51.6	61.1
Nei's haplotype diversity	0.2938	0.3393	0.2481	0.1493	0.2091
Deleterious sites, %	32.5	30.4	26.8	25.2	16.4

<sup>+</sup>Comparison of White and Black Rackas in 2013–2015

Overall, the proportion of deleterious sites (%) appeared to be low (< 30%) and it tended to improve with time, with the exception of the Cikta breed. Furthermore, its value (26.8%) in the Black Racka (2013–2015) appears to be more favourable compared to the White Racka (30.4%; 2013–2015), which is seemingly contradictory to the lower and higher frequencies of haplotypes ARR and ARQ in the former breed. According to the investigations of Ipaté et al. (2013), the calculated proportion of deleterious sites was 26.8% in the 153 Turcana rams studied.

All the fifteen prion genotypes were found in the Milking Tsigai only. In the other breeds the genotypes appeared in lower numbers (Tables 2A and 2B).

**Table 2A**  
Prion genotypes and risk categories (%) of Cikta and Tsigai sheep

Groups	Cikta 2004	Cikta 2013–2015	Tsigai 2004	Tsigai 2013–2015	Milking Tsigai 2013–2015
Genotypes:	(n = 69)	(n = 1145) Chi <sup>2</sup> = 12.564; df = 7; P = 0.083	(n = 64)	(n = 1061) Chi <sup>2</sup> = 51.521; df = 10; P < 0.001	(n = 826)
ARR/ARR	1.45 (1)	2.45 (28)	3.13 (2)	22.05 (234)	7.39 (61)
ARR/AHQ	4.35 (3)	2.79 (32)	6.25 (4)	4.15 (44)	0.85 (7)
ARR/ARH	–	–	0.00 (–)	0.47 (5)	5.08 (42)
ARR/ARQ	33.33 (23)	20.70 (237)	45.31 (29)	42.04 (446)	31.84 (263)
AHQ/AHQ	0.00 (–)	1.31 (15)	0.00 (–)	0.09 (1)	0.00 (–)
AHQ/ARH	–	–	–	–	0.48 (4)
AHQ/ARQ	14.49 (10)	15.98 (183)	0.00 (–)	2.17 (23)	0.97 (8)
ARH/ARH	–	–	–	–	0.48 (4)
ARH/ARQ	0.00 (–)	0.26 (3)	3.13 (2)	0.38 (4)	12.47 (103)
ARQ/ARQ	46.38 (32)	56.42 (646)	40.62 (26)	27.33 (290)	32.57 (269)
ARR/VRQ	–	–	0.00 (–)	0.85 (9)	2.42 (20)
AHQ/VRQ	–	–	0.00 (–)	0.09 (1)	0.24 (2)
ARH/VRQ	–	–	–	–	0.85 (7)
ARQ/VRQ	0.00 (–)	0.09 (1)	1.56 (1)	0.38 (4)	4.24 (35)
VRQ/VRQ	–	–	–	–	0.12 (1)
Risk groups <sup>++</sup> :	(n = 69)	(n = 1145) Chi <sup>2</sup> = 8.846; df = 3; P < 0.031	(n = 64)	(n = 1061) Chi <sup>2</sup> = 26.452; df = 4; P < 0.001	(n = 826)
R1	1.45 (1)	2.45 (28)	3.13 (2)	22.06 (234)	7.39 (61)
R2	37.68 (26)	23.49 (269)	51.56 (33)	46.65 (495)	37.77 (312)
R3	60.87 (42)	73.97 (846)	43.75 (28)	29.97 (318)	46.97 (388)
R4	0.00 (–)	0.00 (–)	0.00 (–)	0.85 (9)	2.42 (20)
R5	0.00 (–)	0.09 (1)	1.56 (1)	0.47 (5)	5.45 (45)

<sup>++</sup>According to risk group correction because ARQ/ARQ was classified as R4 in the past

**Table 2B**  
Prion genotypes and risk categories (%) of Racka sheep

Groups	Racka 2004	White Racka 2013–2015	Black Racka 2013–2015	Gyimes Racka 2004	Gyimes Racka 2013–2015
Genotypes:	(n = 140)	(n = 844) Chi <sup>2</sup> = 38.640; df = 14; P = 0.001	(n = 779) Chi <sup>2</sup> = 64.274; df = 14; P < 0.001 (Chi <sup>2</sup> = 138.359; df = 14; P < 0.001) <sup>+</sup>	(n = 57)	(n = 394) Chi <sup>2</sup> = 110.803; df = 10; P < 0.001
ARR/ARR	5.00 (7)	13.27 (112)	9.37 (73)	5.26 (3)	35.28 (139)
ARR/AHQ	6.43 (9)	13.98 (118)	5.52 (43)	0.00 (–)	3.05 (12)
ARR/ARH	4.29 (6)	9.60 (81)	5.52 (43)	0.00 (–)	2.79 (11)
ARR/ARQ	25.00 (35)	16.59 (140)	30.56 (238)	42.11 (24)	34.26 (135)
AHQ/AHQ	4.29 (6)	6.64 (56)	0.51 (4)	–	–
AHQ/ARH	7.14 (10)	7.11 (60)	1.28 (10)	0.00 (–)	0.25 (1)
AHQ/ARQ	12.14 (17)	8.53 (72)	7.83 (61)	1.75 (1)	2.54 (10)
ARH/ARH	0.71 (1)	2.01 (17)	1.03 (8)	–	–
ARH/ARQ	10.00 (14)	8.53 (72)	8.73 (68)	0.00 (–)	0.76 (3)
ARQ/ARQ	17.86 (25)	6.75 (57)	23.75 (185)	47.38 (27)	14.47 (57)
ARR/VRQ	2.14 (3)	2.25 (19)	1.41 (11)	1.75 (1)	3.30 (13)
AHQ/VRQ	1.43 (2)	0.95 (8)	0.77 (6)	–	–
ARH/VRQ	1.43 (2)	1.42 (12)	0.64 (5)	0.00 (–)	0.25 (1)
ARQ/VRQ	2.14 (3)	2.13 (18)	3.08 (24)	1.75 (1)	3.05 (12)
VRQ/VRQ	0.00 (–)	0.24 (2)	0.00 (–)	–	–
Risk groups <sup>++</sup> :	(n = 140)	(n = 844) Chi <sup>2</sup> = 9.659; df = 4; P = 0.047	(n = 779) Chi <sup>2</sup> = 5.184; df = 4; P = 0.269 (Chi <sup>2</sup> = 2.477; df = 4; P = 0.649) <sup>++</sup>	(n = 57)	(n = 394) Chi <sup>2</sup> = 80.772; df = 4; P < 0.001
R1	5.00 (7)	13.27 (112)	9.37 (73)	5.26 (3)	35.28 (139)
R2	35.71 (50)	40.17 (339)	41.60 (324)	42.11 (24)	40.10 (158)
R3	52.15 (73)	39.57 (334)	43.13 (336)	49.13 (28)	18.02 (71)
R4	2.14 (3)	2.25 (19)	1.41 (11)	1.75 (1)	3.30 (13)
R5	5.00 (7)	4.74 (40)	4.49 (35)	1.75 (1)	3.30 (13)

<sup>++</sup>Comparison of White and Black Rackas in 2013–2015

<sup>++</sup>According to risk group correction because ARQ/ARQ was classified as R4 in the past

Irrespective of the breed the most frequent genotypes were the least favourable ARQ-bearing genotypes. These were followed by the more favourable ARR-bearing genotypes with the exception of the Cikta. The most sensitive homozygous VRQ/VRQ occurred in three individuals only (in Milking Tsigai and White Racka). With regard to the genotypes, there were no significant differences ( $P = 0.083$ ) between the Cikta populations over time, although the frequency of the least favourable genotype increased by 10%. Simultaneously, there were significant improvements in Tsigai and different Racka populations ( $P <$

0.001 in all cases). Viorca et al. (2008) found the ARR/ARR genotype frequency to be 14.63% in 123 males of the Turcana breed [Hatseg (Hațeg) ecotype].

The Chi<sup>2</sup> test proved that all the current populations of Hungarian indigenous sheep breeds (Cikta, Tsigai, Milking Tsigai, White Racka, Black Racka and Gyimes Racka) are in complete Hardy–Weinberg genetic equilibrium ( $P \approx 1.000$  in all cases; the expected frequencies are not presented). A plausible explanation of the genetic equilibrium can be found in the full sampling (all ewes and candidate rams) and the small difference between the expected and observed values. Hardy–Weinberg equilibrium can occur even in the case of five single nucleotide polymorphisms of prion-related protein gene, as it was reported by Kim and Jeong (2017) for the Korean native black goat population.

Risk groups with their changes can be found in the bottom part of Tables 2A and 2B. In the Cikta breed it should be highlighted that risk group R4 is missing and R5 is represented by only one individual. Due to the high frequency of ARQ, R3 is present with almost 74% frequency, and animals that are best suited for breeding (R1) represent only about 2.5%. Between the two consecutive evaluations it was statistically proven ( $P < 0.031$ ) that the Cikta sheep has changed in terms of risk grouping. Improvement can be seen in the increasing rate of R1, but the remarkable increase in the rate of R3 animals is not favourable, especially the increase in the frequency of AHQ haplotype at the expense of ARR (Table 1A). However, in the other breeds which are considered less sensitive than the Cikta there are traces of both risk groups R4 (from 0.85 to 3.30%) and R5 (from 0.47 to 5.45%). Based on the frequency of R1, the greatest improvement or the most efficient response to selection for a scrapie-resistant genotype was achieved in the Gyimes Racka among the Hungarian indigenous sheep breeds.

## Discussion

Our investigation and substantial sampling confirms that the Hungarian indigenous sheep breeds have different genetic resistance to scrapie infection. For the Cikta sheep population this resistance must be rated as low. The risk classification of that breed (especially concerning haplotype ARR) has not improved. At the same time, in the other breeds the response to selection for scrapie resistance was definitely successful. The final aim is to lower the entropy of prion haplotypes through ARR fixing.

It has been found that genic variation may temporarily increase during the selection process. The proportion of deleterious sites (%) can be a useful indicator for the eradication programme.

Possible reasons for differences among the breeds are that the selection for breeding during the last decade was restricted to the rams, and that the short period of time (low number of generations) hindered an intense response.



It is also important to exclude the susceptible animals (with a VRQ allele) from breeding. Efforts should be made to use rams belonging to risk groups 1 or 2. The still frequent haplotype ARQ and risk group 3 in the Cikta breed must be considered as breed specific. Their use is permitted and the genotypes are also applied as markers for the preservation of breed diversity.

The research of Baylis et al. (2004) reassuringly confirmed the considerably lower risk of scrapie in the ARQ/ARQ genotype than in ARQ/VRQ or VRQ/VRQ. From their research it can be strongly concluded that the scrapie risk of homozygous ARQ/ARQ is higher than that of two VRQ-bearing heterozygotes (ARR/VRQ, AHQ/VRQ; there were no statistically significant differences in either case). This suspicion could lead to a more severe selection condition in the Czech breeding programme for scrapie resistance: permission for breeding is given only for ARQ carriers (Stepanek and Horin, 2017).

Future plans include preventing the appearance of scrapie by increasing the use of rams without haplotype VRQ and eliminating VRQ carriers (if they occur). The elimination of AHQ, ARH and ARQ alleles may have a long duration of execution.

Owing to the elimination programme, the proportion of scrapie-resistant sheep (mostly in intensive breeds; Drögemüller et al., 2001) has increased worldwide, evidenced by the increasing frequency of the ARR haplotype. However, in breeds other than the indigenous Hungarian breeds we may encounter even worse and unsatisfactory results. For example, in the investigation of Cameron et al. (2014) the Canadian Arcott breed had higher proportions of susceptible sheep and a higher frequency of VRQ alleles (15% VRQ in a population consisting of 183 individuals) and a higher rate of disadvantageous groups (R4 and R5 over 10% each).

A gradual selection for resistant individuals and in particular for resistant rams should be associated with a reduction in the possible incidence of scrapie. On the other hand, the maintenance of productivity and genetic diversity must also be considered. Some investigations (e.g. those of Nagy et al., 2009) revealed that individuals of improved mutton type carrying the ARR haplotype have a lower daily gain than those without ARR. Álvarez et al. (2007, 2009) concluded that ARR heterozygotes should be chosen first before the beginning of a selective conservation programme, and not all the individuals of undesirable (particularly unacceptable) risk groups (R4 and R5) should be rejected from breeding to avoid the diminishing diversity of autochthonous sheep breeds.

Based on the above arguments, we propose continuation of integrated programmes, such as the National Scrapie Plan for Hungary, with a multiple trait conserving selection of rare breeds.

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