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Genetics of scrapie susceptibility in females of Tsigai Breed

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TABLE OF CONTENTS

1 Introduction and aim

Scrapie is in a group of prion diseases (Transmissible Spongiform encephalopathy), including Bovine Spongiform Encephalopathy's (BSE) in cattle and Creutzfeldt-Jacobs disease of humans. It's a neurological disease caused by a missfolded prion protein (PrP^{SC}), with al long incubation period and a gradual degeneration of the central nervous system.

First it was thought that scrapie was a genetic disease, but studies showed that the genetics of the disease was only associated with susceptibility of the disease only, rather than being a genetic disease. It's proven that different genetic variants have different susceptibility of the infectious disease, and eradication programmes are initiated to reduce the prevalence of susceptibility.

Breeding programmes where they use the more scrapie resistant rams have been used, and proven successful to reducing scrapie susceptibility. It is also proven that it does not interfere with the overall genetic variability of the flocks, at least on a commercial breeding flock.
With this study we will examine if this would also be applicable to a small pool of native Hungarian Tsigai, or if it would impact the gene pool for such a small breeding pool.

Our aim will be to investigate the scrapie resistant and scrapie susceptible haplotypes, genotypes and risk groups in the Hungarian Tsigai population. And at the same time investigate the prevalence's for the ewe population.

2 Review of the literature

2.1 General about scrapie

Scrapie is a fatal neurogenerative disease affecting sheep and goat. It belongs to transmissible spongiform encephalopathies (TSE) together with bovine spongiform encephalopathy (BSE) and the human Creutzfeldt-Jacobs disease. Scrapie belongs to the disease of prions, a fatal slowly degenerative disease. The disease was recognized over 250 years ago in Great Britain and Western Europe (McGowan, 1922). There is a natural form and an atypical, Nor 98 form.

2.2 Clinical signs

Clinical signs frequently starts with a slowly developing behavioral change (Detwiler, 1992). The affected animal may distance itself from the flock, and may display nervous or aggressive behavior. The name Scrapie derived from the appearance of the disease. Animals may present with severe itching, the animals scrape of wool by rubbing against the fence, wall or even pull their own wool. Sheep may also present with pruritus as secondary to itching and scraping (Dickinson, 1976). The atypical form usually do not present with itching, but the remaining signs are similar however the frequency of Clinical signs are decreased (Petter Hopp et al., 2010).

Locomotor problems and ataxia is also a common clinical sign. Trembling, convulsions, blindness and stargazing are also clinical signs associated with scrapie. The condition of scrapie-infected sheep additionally becomes poor. Overall there is a decrease in food intake and decreased rumination in the infected flock (Healy, 2002)
The presence and absence of clinical signs may vary depending on breed, country and strain of scrapie.

The incubation period is long, the sheep get infected when young but clinical signs do not appear till they are mature. Usually the incubation period is between 2-5 years (Choudhary and Choudhary, 2013; O. M. Radostits et al 2006).

2.3 Post mortem findings

There are no significant gross pathological findings. Except from the clinical signs, as wool loss, pruritus and emaciation.

There are however Characteristic histopathological lesions. These include, Neuronal vacuolation, neuronal degeneration and loss, vacuolation of gray matter and neutrophil, astrocytosis and accumulation of amyloid plaques (Detwiler, 1992). Vacuolization of the Gray matter in the spinal cord, medulla, pons and midbrain is the result of accumulation of the PrP^{SC} prion protein (Prusiner, 1996; Wemheuer et al., 2011). The accumulation of the PrP^{SC} may also occur in other tissues such as lymphoreticular tissues (Wemheuer et al., 2011).

It is shown that areas of most concentrated deposition are within the medulla oblongata, brainstem and the Thalamus. Cerebral cortex and Cerebellum were less affected. In the study of E. Vidal et al. they proved that several changes, as involvement of cellular stress molecules (Metallothionein I and II and HSP25) and changes in expression of glial cell and synaptic proteins and aquaporin 1, is also associated with PrP^{SC} accumulation.
The extent of vacuolization can be associated with the different phenotypes, and may also help with the phenotyping of the disease (Ligios et al., 2002).

Amyloid plaques might also appear. Prusiner used Congo red in one of his studies and concluded and the plaque fulfilled the standards for amyloid (Prusiner, 1998). Tests have been carried out in mice, where they show that amyloid plaques may appear before the vacuolization. The plaque in the inoculated mice appeared close to the lateral ventricles, and could conclude that they accumulate close to the PrP^{SC} deposition (Bruce, 1981). The presence of these plaques in sheep is rare, and depends on the different strains (Detwiler, 1992).

2.4 The prion

Scrapie is a prion disease caused by the protein prion PrP^{SC}. The term "prion" comes from Prusiners description of proteinaceous and infectious. Prions are proteinaceous infectious particles without nucleic acid, which in case of scrapie is a missfolding of the PrP^{C} (PrP is short for prion protein) into the PrP^{SC} form of the prion protein. PrP^{C} is a normal protein found normally on the membranes of cells, and is digested by proteinase K enzymes. (Prusiner, 1998)

The PrP^{C} protein is consisting of about 40% and little β-sheets, while the infective PrP^{SC} is missfolded and consists of only 30% α-helixes but about 45% β-Sheets instead (Prusiner, 1998). The β-sheet formation makes the prion protein insoluble and resistant to proteases, while the normal PrP^{C} is broken down by proteases and removed from the membrane surfaces. It's the accumulation of the infective form, due to protease resistance, which makes the pathological changes in Scrapie. The prions accumulate and the degeneration starts. (Eghiaian et al., 2004; McCutcheon et al., 2005; James Foster and Nora Hunter, 1998)

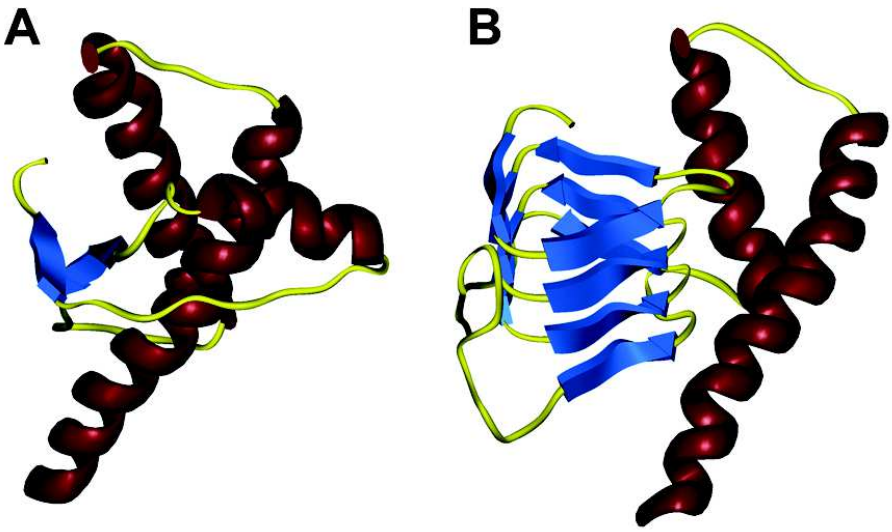

Figure 1: Prion Protein Structure. Conformational changes of normal (A) cellular prion protein (PrP^{C}), into pathological (B) prion protein (PrP^{Sc}).

2.5 Genetic polymorphism in the PrP gene

Based on several studies it is proved that the resistance or susceptibility of scrapie in sheep is influenced significantly by the polymorphism of the PrP (prion protein) gene (PRNP) (Lühken et al., 2008; Bayliss et al., 2000; Hunter, 1997). Several polymorphisms are identified, but there is only a few is linked especially to the occurrence of scrapie.

Resistance or susceptibility is linked to different variations, particularly on codon 136, 154 and 171. These three codons are shown to be the most important in especially natural scrapie (Hunter, 1997; Bayliss et al., 2000; Lühken et al., 2008).

- At codon 136, Alanine (A) is linked to scrapie resistance and valine (V) is related to susceptibility.
- At codon 154, histidine (H) is linked to resistance and arginine (R) is related to susceptibility.
- At codon 171, Arginine (R) is linked to resistance and glutamine (Q) and histidine (H) is related to susceptibility.

After several studies there are five haplotypes that are noticeable. These are the $A_{136}R_{154}Q_{171}$ (ARQ), $A_{135}R_{154}R_{171}$ (ARR), $A_{136}H_{154}Q_{171}$ (AHQ), $A_{136}R_{154}H_{171}$ (ARH), and $V_{136}R_{154}Q_{171}$ (VRQ). The most resistant haplotype is found to be the ARR (Hunter, 1997). And the most susceptible haplotypes are thought to be the ones with homozygous QQ_{171}, especially the VRQ haplotype. For the genotypes the homozygous ARR/ARR genotype will be the most resistant, while the homozygous VRQ/VRQ genotype are the most susceptible (Baylis et al., 2002).

The national scrapie programme (NSP) have made a table (Table 1.) with the 15 most common genotypes, and classified them in 5 groups according to genetic resistance against scrapie. These are used in the breeding programmes for scrapie resistance (DERFA, 2006).

There is an interesting difference in the genotype between different flock and breeds. For example in Suffolk sheep the most resistant genotype is the ARQ (Hunter, 1997). Thereby to be able to investigate and conclude a specific resistance or prevalence for Scrapie, knowledge of the most frequent genotypes in the breed is required (Gesine et al., 2007).

Baylis et al showed us that in scrapie infected flocks there is a high frequency of susceptible genotypes, and a lower frequency of resistant genotypes. They also found that in affected flock there is more susceptible young sheep than Older and susceptible. This is due to the disease and death of the susceptible animals. In Scrapie free flocks there was little difference in genetics in younger and older (Baylis et al., 2000).

In a study to evaluate frequencies of the PrP polymorphism by Lühken et al (2008), in autochthonous European sheep breeds, where amongst other breeds the *Hungarian Tsigai* were sampled. Out of 29 samples there was percentage of 32.76 for the ARR haplotype, but only 13.79 percent for the ARR/ARR genotype. While for the VRQ haplotype there were a percentage of 10.3 while 0 percent of the VRQ/VRQ and a 20.69 percent o the ARQ/VRQ. This study is not big enough to predict the Hungarian Tsigai genotypes, but shows us that it's important to evaluate and check both for haplotype and genotypes (Lühken et al., 2008).

2.6 Transmission of Scrapie

Today it's well accepted that scrapie is a contagious infectious disease, not a genetic disease. However the genetics is a factor in which individual who is more easily infected with the disease (Foster and Hunter, 1998). As a contagious disease scrapie also have several modes of transmission; we have lateral (horizontal), vertical and maternal transmission.

Lateral transmission is the spread of infection between animals either directly by contact or indirect. The scrapie agent has been detected in Nervous system, brain, salivary glands, tonsils, lymph nodes, spleen, colon, placenta and many other organs which all may be a possible transmission source. The placenta is considered the major spread of the disease, either to their own lambs or to other animals in close contact (Ryder et al., 2003). Hourrigan and Klingsporn (1996) also proved that the longer the lambs was in contact with the contaminated environment, were more likely to contract the disease. Ryder et al (2003) also confirmed the infection between infected sheep and both offspring born from the infected ewes, and also lambs and adults introduced to the flock. Studies have also presented that the younger the lamb is, the easier they are infected. Its suggested that the reason is the gut environment of neonatal lambs are more favorable, and also the

macromolecular absorption is more efficient in younger than adults (Hunter et al., 2012). Transmission via milk is also researched and it is demonstrated that the disease could be transmitted via the milk, and also shed to the environment after infection (Konold et al., 2008).

Lateral transmission can also be between sheep in close contact with infected environment (Konold et al., 2015; Detwiler, 1992). A study using contaminated furniture and water troughs demonstrates the transmission from contaminated equipment to susceptible sheep. Since the disinfection and decontamination of contaminated equipment and furniture is not sufficient to remove scrapie infectivity, the conclusion is to recommend removal and disposal contaminated equipment (Konold et al., 2015)

Iatrogenic transmission is also a way of spreading. Evidence proved that accidental transmission via vaccines might occur, although it's a rare incident. But an outbreak in 2001 with scrapie was epidemiologically connected to the vaccinations against mycoplasma agalactia (Caramelli et al 2001).

Vertical transmission is explained by the genetic transmission of the disease. In scrapie it is associated with the transmission to offspring via germ plasma either during embryonic development or at the time of fertilization (Detwiler, 1992; Detwiler and Baylis, 2003). The ram plays a minor role in transmission compared to ewes. Transmission via semen is exceptionally low but it cannot be ruled out yet, more research is needed to completely rule out the form of transmission (Rubenstein et al., 2012; Detwiler and Baylis, 2003). The vertical transmission from the ewe in utero is also occurring. After embryo transfer and cesarean section, to avoid infection from placenta at birth, lambs were still infected with scrapie (Foster et al., 2013; Spiropoulos et al., 2014).

Maternal transmission is defined as vertical or lateral transmission from the ewe to its offspring. It's usually connected to transmission via the placenta, which can accumulate large amounts of prions in incubating animals. This is the major source of infection from mother to lamb, and also lambs in close contact (Detwiler and Baylis, 2003; Foster et al., 2013, Spiropoulos et al., 2014). Thus it's recommended to remove placentas from the pen as soon as possible to decrease the source of infection. The placentas should be securely disposed of, not composted, to prevent any further transmission (Healy et al., 2004).

2.7 Diagnosis

The specific causative agent, for all prion disease, have not been isolated or described. This makes the diagnosis more difficult and it must be relied on phenotypic parameters, such as clinical signs, histopathological profiles, complex immunochemical and biological parameters (OIE manual, 2012).

The diagnosis depends on histopathological examination for the detection of pathological changes associated with scrapie. The most reliable and appropriate diagnosis is done by histopathology in a section of medulla oblongata taken at the level of the obex. Nevertheless there are supplying methods for detection of disease-specific forms of PrP using Immunohistochemical methods (IHC), Western immunoblot methods, rapid test methods and other diagnostic tests (OIE manual, 2012).

In 2013 the surveillance programme according to the European union regulations, Regulation (EC) No 999/2001 Annex III, with amendments and including the examination of the following categories of sheep:

- 10 000 slaughtered sheep over 18 months
    - Healthy animals slaughtered for human consumption
        - A minimum sample of healthy animals over 18 months
- 10 000 dead sheep over 18 months
    - Animals not slaughtered for human consumption
        - Fallen stock which showed clinical signs ante mortem, died or killed but not in framework of an TSE epidemic
    - Animals culled under TSE eradication
    - Animals clinically suspected of being infected

2.8 Reduction of prevalence

Scrapie susceptibility is as explained earlier connected to the genetic makeup of the animals. Genetic testing will allow the selection of resistant genes for further breeding and culling of the most susceptible sheep. Breeding programs, which use rams with the resistant ARR gene, have proven to be effective in reducing occurrence of the susceptible gene (Nodelijk et al., 2011). In the Netherlands the genetic-testing of rams have been compulsory from 2004-2007, and in this period only ARR rams have been used for breeding. The results have been good; there is an increase in resistant genes, and also lower incident of scrapie cases (Melchior et al., 2010).

In dairy sheep flocks milk tank samples can also be used for determining the genetic composition in a sheep flock, and have a good potential to be applied in breeding programs. Together with the ram genotyping it could enhance the efficiency of the breeding program for scrapie resistance (Psifidi et al., 2013).

Some may be worried that breeding for scrapie resistance may cause little genetic variability and bottlenecks in the gene pool. A French study was done on four different breeds of different stock size and different variety of genetic traits. During this study they manage to increase the appearance of the ARR allele, and no red flags were raised when it came to population genetics and variability. But it's still uncertain if it's too early to notice any effective reduction in population size (Palhiere et al., 2008)

2.9 Eradication

Eradication programmes of transmissible spongiform encephalopathies in the European Union made and set by the European Commission. Regulation (EC) No 999/2001 lays down rules for prevention, control and eradication of transmissible spongiform encephalopathies.

In Hungary breeding programmes for scrapie resistance have been done according to the Regulation (EC) No 999/2001. Fésüs and associates carried out a genotyping programme for sheep prion protein in 2004-2006. They used 10 commercial and 4 indigenous (Gyimes Racka, Hortobagy Racka, Tsigai, Cikta) breeds, to evaluate the preliminary results of the breeding programme. Accordingly they used the NSP (National scrapie programme) genotype table (table 1.) to decide the degree of resistance or susceptibility, the NSP1-NSP3 Genotypes will be approved for breeding (Fésüs et al., 2008).

According to the Hungarian national breeding programme all rams should be genotyped, and only NSP 1-3 rams are allowed to be bred, and preferably avoiding the NSP3 rams if possible. Animals with VRQ allele may leave the flock only for slaughter (European commission, 2012). An exception in the breeding programme is the indigenous breeds, the Tsigai falls under this Indigenous category. Which means that non-tested rams also can be used.

After the evaluation of Fésüs and associates the increase of the ARR allele can clearly be seen, except in Charolaise and British Milk sheep. They concluded that it might be more efficient to breed for ARR allele in smaller breed populations (Fésüs et al., 2008).

Table 1. NSP risk grading table

Risk group	Genotype	Degree of resistance/susceptibility
NSP1	ARR/ARR	Sheep that is genetically most resistant to scrapie.
NSP2	ARR/AHQ ARR/ARH ARR/ARQ	Sheep that are genetically most resistant to scrapie, but will need careful selection when used for further breeding.
NSP3	AHQ/AHQ AHQ/ARH AHQ/ARQ ARH/ARH ARH/ARQ ARQ/ARQ	Sheep that genetically have little resistance to scrapie and will need careful selection when used for further breeding.
NSP4	ARR/VRQ	Sheep that are genetically susceptible to scrapie and should not be used for breeding unless in the context of a controlled breeding program.
NSP5	AHQ/VRQ ARH/VRQ ARQ/VRQ VRQ/VRQ	Sheep that are highly susceptible to scrapie and should not be used for breeding.

Note: information collected from DERFA (2006)

3 Materials and methods

3.1 Sheep

The Hungarian Tsigai

The Tsigai is an ancient independent and highly developed breed, originated in Asia Minor and came to Hungary in the 1700. In 1880 the merino was introduced, because of its higher production value, and the Tsigai population started to decrease (Bodó I et al. 2000).

Figure 2. Group of freshly lambed ewes under year round free-range condition in Pénzesgyőr (Photo A. Gáspárdy, 2012)

It is a medium sized, long tailed breed, usually with white wool color, and black extremities and face, although different color variant may appear. The white wool may appear gray due to their dispersed black fibers.

At the moment we have two types, the mutton type and the milking type Tsigai. The milking type may produce up to 40-60 liters in a 4 months milking period, and the mutton type is known for its excellent meat quality. They are sturdy animals and an adult ewe is

about 50-55kg with wither heights of 65-70 cm. Their head is convex and the udder is well developed. Some of the rams have horns, however polled rams also frequently appear. The lambs are large and they develop quickly. Lambs are usually born in a darker color, brown, dark brown, dark grey, but becomes white after a short period (Bodó I et al. 2000).

Figure 3. Tsigai ewes in shelter at the farm in Kúnfehértó
(Photo A. Gáspárdy, 2014)

The breed has a high significance within the Hungarian culture and the preservation of the breed is of great importance, in spite of a lower production value than other commercial breeds. In year 2000 there was only 50 rams and 1000 ewes registered in the seed stock managed by National Parks of Kirkunsag and Körös-Maros. In addition there are about 3000 animals dispersed throughout the country (Bodó I et al. 2000).

3.2 Case selection

Data and results from the genetic tests for scrapie are collected from the database of Hungarian Association of Sheep and Goat Breeders (MJKSZ). The data is collected from females and male Tsigai sheep in Hungary in the summer of 2015.

Parameters investigated were scrapie prion Haplotypes, genotype and risk factor. They were counted and their frequencies calculated and compared. We found all the allelic haplotypes, however not all of the genotypes were present in the Tsigai population. By this means we had to count the missing genotypes as 0 observations.

In total we have samples from 392 Mutton Tsigai, 93 female and 299 males. These are collected from all together 19 Tsigai farms all over Hungary. Both smaller and larger farms were tested to get a good diversity of the population.
For the milking Tsigai, there was only 8 male sheep tested, thereby we didn't have enough samples for a complete analysis (Table 3.). In our tables n=sample size.

3.3 Sampling

Samples are collected by instructors from the Hungarian Association of Sheep and Goat Breeders (MJKSZ) based regionally. Special pliers (Figure 4.) are used to take the samples from the cartilaginous tissue of the auricles, the TypiFix^{TM} system developed by the German company Agrobiogen. Prices per sample are 24 EUR, and the samples are paid by MJKSZ. The pedigree information and hard book data are also stored by MJKSZ (Gáspárdy, A. and L. Sáfár, 2015).

From 2006 Hungary sends the samples to the German company Agrobiogen. The samples are sent and analysis prepared, and the results are received in only one month. Before this time the samples were evaluated at the Research Institute for Animal breeding and Nutrition (ATK), Herceghalom (Hungary).

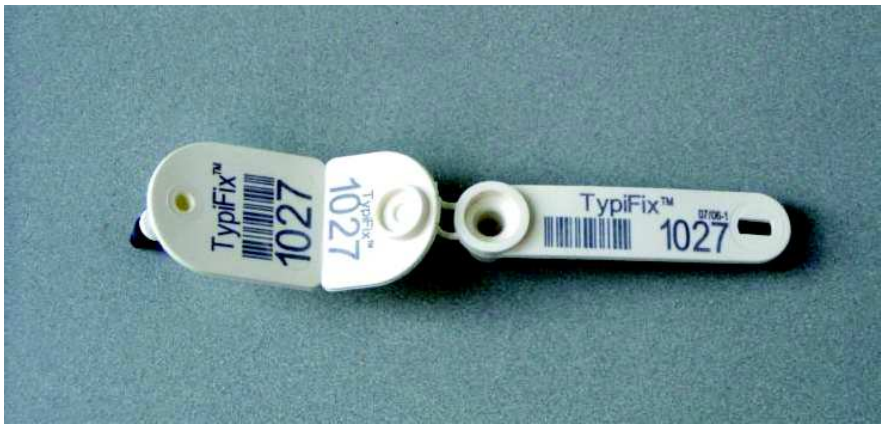

Figure 3. TypiFix™ ear-tag

The TypiFix™ (Figure 3.) ear tag system is a conventional system with an integrated tissue sample technology. When tagging the animals the spike of the ear tag takes a tissue sample. Samples are collected in a special container where the conservation of the samples begins immediately. The ear tag system also makes it easier to read and number the samples correctly. The TypiFix™ can also work without ear tags, only sampling. The easy handling of the TypiFix™ allows safe and economic sampling of the whole population, without the need of veterinarians to take blood samples (Agrobiogen, 2004).

Figure 4. TypiFix™ pliers. Photo A. Gáspárdy, 2011

3.4 Laboratory analysis

First, a fragment of the prion protein gene is amplified in vitro by means of the polymerase chain reaction (PCR). Then, the base sequence/composition of the amplified genetic material (DNA) is determined by sequencing. By this more genetic information is obtained. By this no extra effort is needed for the reliable detection of rare mutations such as the T variant at codon 136 and the K variant of codon 171 (Bogdan et al 2009, Agrobiogen 2016)

When scrapie genotyping with the TypiFix^{TM} method, the samples go trough a simple one-step DNA purification with DNA FIX columns. Sorbents retain protein and other contaminants, while the DNA passes the column in the exclusion volume (Bogdan et al 2009, Agrobiogen 2016).

An automatic pipetting robot and a special one-step procedure preform DNA isolation and purification. PCR samples can also be automatically prepared. The Samples are then compared to Primers to find the correct allelic frequencies. The results of the PCR is linked to the identification number on the sample and ear-tag, which makes it easier ta scan and save in the animal data bank. This aspect of the TypiFix^{TM} system is important for traceability and domestic animal biodiversity (Bogdan et al 2009, Agrobiogen 2016).

3.5 Statistics

Statistics were preformed with the statistical analysis programme Dell statistica (Dell Inc. 2015), and with data collected in Microsoft Excel from the database of Hungarian Association of Sheep and Goat Breeders.

We compared the overall statistical data, the whole population, males and females separately and compared. Also a comparison with previously collected data was made. The difference between expected and observed, the relative frequency, Chi-square test and calculation of the Hardy Weinberg equilibrium (HWE) were preformed. Chi-square test was used to assess the differences between categorical variables of the current study and previously published studies, and similarly Males versus Females. And in case of the genotypes (where some of the genotypes were not present) we used the chi-square test for control of the Hardy Weinberg Equilibrium. Hardy Weinberg Equilibrium is used to see if

the distribution of genotypes were compatible with the Hardy Weinberg Equilibrium. Chi-squared test performed should have $p<0,001$ (probability) to be compatible, which means that there isn't any deviation from the Hardy Weinberg Equilibrium.

Statistical test was done on the counted number of observations. Chi-squared test was used. Here our observed numbers were compared to expected numbers:

- To see differences in frequencies of categories
- To see the genetic equilibrium stage (HWE)
- To see differences between males and females in 2015
- To see differences between present (2015) and past (2004)

3.6 Literature data analysis

The data gathered in this current study was compared to the data collected and published in a previous study, Breeding for scrapie resistance in the Hungarian sheep population, by Fésüs et al. 2008. Where they gathered data of PrP genotypes in commercial and indigenous breeds in the years from 2004-2006, the Tsigai were included in the four indigenous breeds.

4 Results and discussion

In all 392 samples from 2015 were appropriate for our investigation (table 2.), there were 5 incomplete samples, in addition to the samples from the milking Tsigai which were not submitted in our investigation.

Table 2. Short summary of data collected.

Haplotypes observed ARR AHQ ARH ARQ VRQ			
Categories	**Total number** tested n=392	**Males tested** n=299	**Females tested** n=93
Genotypes observed			
ARR/ARR	105	78	27
ARR/AHQ	22	12	10
ARR/ARH	2	1	1
ARR/ARQ	163	124	39
AHQ/ARQ	9	4	5
ARQ/ARQ	83	73	10
ARR/VRQ	6	5	1
VRQ/ARQ	2	2	0

Note: Data collected from MJKSZ

As seen in the data from the milking Tsigai (Table 3.), there is not enough information to produce a complete analysis. These results were additionally from one and the same farm, which does not give a proper coverage of Hungary.

Table 3. Summary of milking Tsigai results

Genotype	Number observed males	Risk category
ARR/ARR	3	R1
ARR/ARQ	1	R2
ARQ/ARQ	1	R3
ARR/VRQ	1	R4
ARQ/VRQ	2	R5

Note: Data collected from MJKSZ

4.1 Evaluation of allele-, genotype- and risk category variants for evenness in the male and female share population

Table 4. Evaluation of allele-, genotype-, and risk category variants for evenness in the male and female share population

Categories	No of observations, Males	No of observations, Females	Observed relative frequencies, Males	Observed relative frequencies, Females	Male/Females Expected relative frequencies	Partial Chi-square values, p-values, Males	Partial Chi-square values, p-values, Females
Haplotypes	2n=477	2n=149					df=4
ARR	220	78	0.4922	0.5235	0.2000	190.79	77.96
AHQ	16	15	0.0358	0.1007	0.2000	60.26	7.35
ARH	1	1	0.0022	0.0067	0.2000	87.41	27.83
ARQ	230	54	0.4541	0.3624	0.2000	144.35	19.65
VRQ	7	1	0.0157	0.0067	0.2000	75.95	27.83
						Total= 558.76 p<0.001	*Total=160.63 p<0.001*
Genotypes	*n=299*	*n=93*					df=14
ARR/ARR	78	27	0.2609	0.2903	0.0666	169.15	69.78
ARR/AHQ	12	10	0.0401	0.1075	0.0666	3.16	2.33
ARR/ARH	1	1	0.0033	0.0108	0.0666	17.98	4.36
ARR/ARQ	124	39	0.4147	0.4194	0.0666	543.31	173.52
AHQ/AHQ	0	0	0.0000	0.0000	0.0666	19.93	6.20
AHQ/ARH	0	0	0.0000	0.0000	0.0666	19.93	6.20
AHQ/ARQ	4	5	0.0134	0.0538	0.0666	12.47	0.23
ARH/ARH	0	0	0.0000	0.0000	0.0666	19.93	6.20
ARH/ARQ	0	0	0.0000	0.0000	0.0666	19.93	6.20
ARQ/ARQ	73	10	0.2441	0.1075	0.0666	141.27	2.33
ARR/VRQ	5	1	0.0167	0.0108	0.0666	11.19	4.36
AHQ/VRQ	0	0	0.0000	0.0000	0.0666	19.93	6.20
ARH/VRQ	0	0	0.0000	0.0000	0.0666	19.93	6.20
VRQ/ARQ	2	0	0.0067	0.0000	0.0666	16.13	6.20
VRQ/VRQ	0	0	0.0000	0.0000	0.0666	19.93	6.20
						Total=1054.4633 p<0.001	*Total=306.5161 p<0.001*
Risk-group	n=299	n=93					df=4
R1	78	27	0.2609	0,2903	0.2000	5.54	3.79
R2	137	50	0.4582	0,5376	0.2000	99.66	53.01
R3	77	15	0.2575	0,1613	0.2000	4.95	0.70
R4	5	1	0.0167	0,0108	0.2000	50.22	16.65
R5	2	0	0.0067	0,0000	0.2000	55.87	18.60
						Total=216.23 p<0.001	Total= 92.75 p<0.001

From the comparison table for males and females (Table 4.) we can conclude that the relative frequencies are quite similar. And also when looking at the risk groups the Relative frequencies of R4-R5 is rather low, which is encouraging for the breeding of resistant genotypes. For the female group we also found that there's a highest occurrence of the resistant haplotype ARR, while Males have a slightly higher occurrence of the ARQ and second most on the ARR haplotype. This means that in the Hungarian Tsigai population there is a higher frequency of resistant than susceptible individuals, but a slightly higher observation of the resistant ARR haplotype in females. This even if its only the breeding males tested, which gives us reason to expect a higher frequency if breeding with tested ewes as well.

Figure 6. Ram auction at the Centralised Ram Station in Mezőhegyes – rams of R1 or R2 risk category get qualified for further breeding. (Photo A. Gáspárdy, 2012)

4.2 Evaluation of allele-, genotype and risk category variants for evenness in the whole population

Table 5. Evaluation of allele-, genotype and risk category variants for evenness in the whole population

Categories	No of observations	Observed relative frequencies	Expected relative frequencies	Partial Chi-squared values
Alleles	2n=596			df=4
ARR	298	0.5000	0.2000	268.00
AHQ	31	0.0520	0.2000	65.26
ARH	2	0.0034	0.2000	115.23
ARQ	257	0.4312	0.2000	159.30
VRQ	8	0.0134	0.2000	103.74
				Total= 711.7349 p=0.004
Genotypes	n=392			df=14
ARR/ARR	105	0.2679	0.0666	238.01
ARR/AHQ	22	0.0561	0.0666	00.65
ARR/ARH	2	0.0051	0.0666	22.29
ARR/ARQ	163	0.4158	0.0666	716.81
AHQ/AHQ	0	0.0000	0.0666	26.13
AHQ/ARH	0	0.0000	0.0666	26.13
AHQ/ARQ	9	0.0230	0.0666	11.23
ARH/ARH	0	0.0000	0.0666	26.13
ARH/ARQ	0	0.0000	0.0666	26.13
ARQ/ARQ	83	0.2117	0.0666	123.74
ARR/VRQ	6	0.0153	0.0666	15.51
AHQ/VRQ	0	0.0000	0.0666	26.13
ARH/VRQ	0	0.0000	0.0666	26.13
VRQ/ARQ	2	0.0051	0.0666	22.29
VRQ/VRQ	0	0.0000	0.0666	26.13
				Total= 1333,4609 p<0.001
Risk Group	n=392			df=4
R1	105	0,2679	0.2000	9.03
R2	187	0,4770	0.2000	150.43
R3	92	0,2347	0.2000	2.36
R4	6	0,0153	0.2000	66.86
R5	2	0,0051	0.2000	74.45
				Total= 303.1276 p=0.185

In the results from the total Tsigai population (Table 5.), we can see the population as a whole. For the ARR haplotype we have the highest frequency, while for the VRQ haplotypes we have a quite low frequency. The risk groups 1-2(3) also have the highest occurrence, which is higher than our expected values. This is a positive result, and the frequency of scrapie susceptible Tsigai is remarkably lower than the resistant genotypes.

Table 6. The control of the Hardy Weinberg's equilibrium in the whole population

Genotypes	Relative frequency expected	Relative frequency observed
ARR/ARR	25.0000	26.7857
AHQ/ARR	5.2013	5.6122
ARH/ARR	0.3356	0.5102
ARR/ARQ	43.1208	41.5816
AHQ/AHQ	0.2705	0.0000
AHQ/ARH	0.0349	0.0000
AHQ/ARQ	4.4857	2.2959
ARH/ARH	0.0011	0.0000
ARH/ARQ	0.2894	0.0000
ARQ/ARQ	18.5940	21.1735
ARR/VRQ	1.3423	1.5306
AHQ/VRQ	0.1396	0.0000
ARH/VRQ	0.0090	0.0000
ARQ/VRQ	1.1577	0.5102
VRQ/VRQ	0.0180	0.0000
Chi-square=2.8837 df=14 p=0.99		

The *Hardy Weinberg's equilibrium* was used to see if our distribution of genotypes were compatible with Hardy Weinberg's Law (HWL), the chi-squared test preformed should be $p<0.001$, values over 0.001 shows us a low distribution which would be unfortunate for our results. The p-value in our results is low, and after checking the chi-square test with the Hardy Weinberg's equilibrium, we got a p-value on 0.999, which are almost 1.00. This means that our results and calculations are compatible with HWL.

4.3 Comparison of allele-, genotype- and risk category variants of past and present populations

Table 7. Comparison of allele-, genotype- and risk category variants of past and present populations

Components	Observed frequencies in the Present	Observed frequencies in the Past	Difference	Chi-square components
Haplotypes	n=596	n=64		df=4
ARR	50.00	28.90	-21.1	15.4052
AHQ	5.20	3.13	-2.1	1.3708
ARH	0.34	1.56	1.2	0.9610
ARQ	43.12	65.63	22.5	7.7200
VRQ	1.34	0.78	-0.6	0.4053
				Total= 25.8623 p<0.001
Genotypes	n=329	n=64		df=14
ARR/ARR	26.79	3.13	-23.7	178.7836
ARR/AHQ	5.61	6.25	0.6	0.0651
ARR/ARH	0.51	0.00	-0.5	-
ARR/ARQ	41.58	45.30	3.7	0.3052
AHQ/AHQ	0.00	0.00	0.0	-
AHQ/ARH	0.00	0.00	0.0	-
AHQ/ARQ	2.30	0.00	-2.3	-
ARH/ARH	0.00	0.00	0.0	-
ARH/ARQ	0.00	3.13	3.1	3.1300
ARQ/ARQ	21.17	40.63	19.5	9.3172
ARR/VRQ	1.53	0.00	-1.5	-
AHQ/VRQ	0.00	0.00	0.0	-
ARH/VRQ	0.00	0.00	0.0	-
ARQ/VRQ	0.51	1.56	1.0	0.7065
VRQ/VRQ	0.00	0.00	0.0	-
				Not estimable
Risk categories	n=329	n=64		df=4
R1	26.79	3.13	-23.7	178,7836
R2	47.70	6.25	-41.5	274,9505
R3	23.47	45.30	21.8	10,5204
R4	1.53	43.76	42.2	40,7523
R5	0.51	1.56	1.0	0,7065
				Total=505.7134 p<0.001

Note: Data collected from MJKSZ, Fésüs et al 2007 and Fésüs et al 2004.

Comparing the present results and the past results (Table 7.), a significant difference can be seen. As seen in the table (Table 7.) some of the chi-square values were too small and the p-value not estimable, but from the present values we have a good foundation to make a conclusion. In the past results from 2004 there is a highest frequency of the ARQ haplotype and following the ARR haplotype. The present results show a higher frequency in the ARR haplotype while the ARQ haplotype is following. For the Genotypes and risk groups there were in the past a significantly higher frequency of Risk group 3-4, which now should be avoided in the breeding programme. The present results of genotypes and risk group show a shift towards risk group 1-2. These results show a positive evolution for the scrapie resistance. Even if the numbers of tested animals were less in 2004 we have a good indication of progress in the populations scrapie resistance.

4.4 Comparison of allele-, genotype- and risk category variants of male and female share populations 2015

In table 8 we have made a comparison for overview of the observed frequencies for males and females and the difference between the observed results. In the table we have also here some too low chi-square values and a non-estimable p-value, but from the existing values we have a good foundation to make a conclusion. As seen previously it's clearly a slightly elevated frequency in females for the more resistant haplotypes, genotypes and risk categories, seen as a negative difference in the resistant and positive difference for the more susceptible. The biggest difference we have is in the ARQ haplotype with 9.2 in difference, the ARQ/ARQ genotype and in the R3 risk group. These differences also support the conclusion with a slightly more resistant female population.

Table 8. Comparison of allele-, genotype- and risk category variants of male and female share populations 2015

Components	Observed frequencies in males	Observed frequencies in females	Difference	Chi-square components
Haplotypes	n=596			df=4
ARR	49.22	52.35	-3.1	0.199
AHQ	3.58	10.07	-6.5	11.765
ARH	0.22	0.67	-0.5	0.925
ARQ	45.41	36.24	9.2	1.852
VRQ	1.57	0.67	0.9	0.516
				Total= 15.217 p=0.004
Genotypes	n=392			df=14
ARR/ARR	26.09	29.03	-2.9	0.331
ARR/AHQ	4.01	10.75	-6.7	11.3296
ARR/ARH	0.33	1.08	-0.8	1.705
ARR/ARQ	41.47	41.94	-0.5	0.005
AHQ/AHQ	0.00	0.00	0	-
AHQ/ARH	0.00	0.00	0	-
AHQ/ARQ	0.0134	5.38	-4.0	12.180
ARH/ARH	0.00	0.00	0	-
ARH/ARQ	0.00	0.00	0	-
ARQ/ARQ	24.41	10.75	13.7	7.644
ARR/VRQ	1.67	1.08	0.6	0.208
AHQ/VRQ	0.00	0.00	0	-
ARH/VRQ	0.00	0.00	0	-
ARQ/VRQ	0.67	0.00	0.7	0.670
VRQ/VRQ	0.00	0.00	0	-
				Not estimable
Risk categories	n=392			df=4
R1	26.09	29.03	-2.9	0.331
R2	45.82	53.76	-7.9	1.376
R3	25.75	16.13	9.6	3.594
R4	1.67	1.08	0.6	0.208
R5	0.67	0.00	0.7	0.670
				Total=6.180 p=0.186

5 Conclusion and recommendations

5.1 Conclusion

In our current study we have compared males and females and also total population from 2015 to 2004. All over we are pleased with our results, which were more optimistic than originally suspected. We already suspected that the susceptible genotypes would be in a lesser frequency after reviewing the study by Lühken et al., 2008.

For the female population we found for the haplotypes that the highest frequency were of the most resistant allele combination ARR. The second highest number of observations was the ARQ haplotype, which are more scrapie susceptible. With the highest observation of the ARR haplotype we could assume that there were more genotypes with the ARR. The homozygous ARR/ARR genotype, in Risk group 1, was observed as the second highest frequency. While the ARR/ARQ, from risk group 2, were the highest. The genotypic observation for the highest risk group was the ARR/VRQ genotype from the risk category 4.

From these results for female group we could conclude with a significantly lower prevalence of the susceptible haplo- and genotypes. Showing these results there are no danger in using only the most resistant risk group (risk group 1-2) for further breeding, particularly when the prevalence of scrapie susceptible ewes are so low. There will not be any significant load on the overall gene pool since the resistant genetics already are in majority.

Table 9. Male vs. Females in relative frequency of haplotypes

Haplotypes	Relative frequency, Observed males	Relative frequency, Observed females
ARR	0.4921	0.5235
AHQ	0.0357	0.1007
ARH	0.0022	0.0067
ARQ	0.4541	0.3624
VRQ	0.0156	0.0067

When looking at the male population we have similar frequencies as for the females, even if there are a higher total number of observations in males. There are however a higher frequency of the ARQ/ARQ genotype, which gives a significantly higher percentage of males in risk group 3. In the breeding programmes there is an exemption for the use of only risk group 1-2 in breeding for the indigenous breeds. Since Tsigai is in this exemption category non-tested males are still used in the breeding, which may have an influence in our results.

In spite of everything when looking at the haplotypes (Table 9.) there are similar results for the males and females. This demonstrates the importance of checking the genotypes even if there is a majority of the ARR haplotype.

Comparing the Present results with the past results from Fésüs et al 2008, our total (males and females) present study is compared (Table 7.). In our study we observed a percent as high as 50% of the ARR haplotype, 42.12% of the ARQ haplotype and as low as 1.34% of the VRQ haplotype. In the past study by Fésüs et al 2008, we have a different distribution with 28.90% ARR, 65.63% ARQ and 0.78% of the VRQ haplotype. There is a positive shift in the ARR haplotype from 2004 to 2015.

For the homozygous ARR/ARR genotype we observed a percentage of 26.79 % in 2015, compared to the 3.13% in 2004. There is also a considerable difference in the homozygous ARQ/ARQ genotype from 40.63% in 2004 to only 21.17% in 2015. Simultaneously we have an increased percentage in risk group 1-3 in the current study. R1 is 26.9%, R2 is 47.70% and R3 is 23.47%. While in the present study there is a highest percentage in risk group 3-4, R3 is 45.30% and R4 is 43.76%. By these results we can prove that there is a certain progress towards a more scrapie resistant Tsigai gene pool. This is possibly due to the breeding programme for scrapie resistance plus the fact that there is no scrapie-present in Hungary nowadays; this also gives shift towards natural resistance as well.

5.2 Recommendations

The relative frequencies compared shows that there is an equal distribution between the male and female Tsigai. This means that there is no excessive pushing of the genetics when selecting for the more resistant rams. Still there are many possibilities for improvement of the breeding programme for scrapie resistance. Since the Tsigai is an indigenous breed, there is still use of untested rams. By being consistent on using less susceptible/approved rams, the resistance will be even more prevalent.

Since there is already a higher frequency of the more resistant genotypes in females, it could be an additional improvement to use tested ewes also. By breeding risk group 1 and 2 rams with risk group 1 and 2 ewes there will be an improvement in the resistant genotypes. By carefully planning the breeding it may be possible to exclude the most susceptible genotypes. And by our results in this study there will not be any significant change in the population by choosing the more resistant genotypes for breeding. It would also be preferable to test a higher number of individuals. Since the scrapie resistant testing is subsidized by MJKSZ this is a possibility for farmers to do so without it being economically inconvenient. And in case of any scrapie outbreaks, the Tsigai could be more resistant and thereby it would be easier to preserve the native population.

6 Summary

In this study we examined the prevalence of scrapie resistant and susceptible haplotypes and genotypes in Hungarian Tsigai sheep. The reason for the study is to examine the frequencies of the different genotypes, and to find out if there is some improvement in the prevalence of susceptible compared to resistant genotypes through the years. We also put weight on the female population, since the breeding programmes focus most on the rams not the ewes. The results also show if it is possible to breed for the most resistant genotypes, without depleting the total genetic pool of the Tsigai.

To get a general overview of the genetics and to get the individual haplotypes, we received this information from the Hungarian Association of Sheep and Goat Breeders (MJKSZ) to get a better understanding of the overall results of the Hungarian Tsigai.

In our study we found a higher prevalence overall in the more scrapie resistant genotypes. Whereas in the susceptible genotype group, there were only a few individuals found. Moreover in the ewes we would suspect, due to no specific ewe-breeding programme, a higher prevalence of the scrapie susceptible genetics, but correspondingly in the ewes we found a higher frequency of resistant types.

Compared with results from Fésüs et al 2008, the results show a positive development towards a higher prevalence of more scrapie resistant genotypes.

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9 List of tables and figures

9.1 Tables

9.2 Figures