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To cite this article: Ludovica Curcio, Emiliano Lasagna, Francesca M. Sarti, Carla Sebastiani, Giovanni Pezzotti & Massimo Biagetti (2015) Biodiversity and Genetic Polymorphisms Against Scrapie in Sopravissana Sheep Breed, Italian Journal of Animal Science, 14:4, 4251

To link to this article: <http://dx.doi.org/10.4081/ijas.2015.4251>



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Published online: 14 Mar 2016.



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## SHORT COMMUNICATON

## Biodiversity and genetic polymorphisms against scrapie in *Sopravissana* sheep breed

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### Abstract

Scrapie is a neurodegenerative disease affecting ovine and it is one of several transmissible spongiform encephalopathies (TSEs). Scrapie is recognized as two forms – classical and atypical. Susceptibility or resistance to classical scrapie is strongly regulated by the polymorphisms at codons 136, 154 and 171 of the *PRNP* gene. Genetic role in atypical scrapie has been described at codons 141 and 154, these ones are involved in the occurrence of the disease at different risk degrees. The aim of this study was to assess the allelic and genotypic frequencies in *Sopravissana* breed, an endangered autochthon breed of central Italy; in addition, the presence and the frequency of potential protective ARQ allele variants able to increase scrapie resistance by preserving a higher variability of *PRNP* were evaluated. Three alleles (ARQ, ARR and AHQ) and seven genotypes were observed based on codons 136, 154 and 171 with different frequencies. Moreover, the entire coding sequence of prion protein (*PRNP*) of the ARQ/ARQ was sequenced; two non-synonymous L141F (23.8%) and H143R (16.7%), also two synonymous polymorphisms (codons 231 and 237) were found. The results showed that the ARQ/ARQ sheep should be considered as a genetic class, which may potentially include animals with different scrapie susceptibility because of the presence of additional polymorphisms. This may allow the application of alternative strategies for breeding programmes in this endangered breed.

### Introduction

Scrapie, a neurodegenerative disease affecting sheep and goats, is one of several transmissible spongiform encephalopathies (TSEs) or prion diseases. The disease is characterized by the accumulation of an abnormal isoform (PrP<sup>Sc</sup>) of a host-encoded cellular prion protein (PrP<sup>C</sup>) in the central nervous system (Hunter, 1997). Since 1990s, data began to emerge about a role of genetics in the etiopathogenesis of scrapie. The role of different strains of scrapie in influencing the genetic susceptibility of small ruminants to scrapie has been known for a long time (Goldmann, 2008).

It has been now widely demonstrated that the gene coding for the prion protein carries several single nucleotide polymorphisms (SNPs) able to influence susceptibility of animals to classical scrapie. The detection of polymorphisms at codons 136, 154, and 171 suggests that VRQ is associated with a high incidence of scrapie, and ARR is associated with a low incidence of scrapie (Baylis *et al.*, 2004). Moreover, in atypical form of scrapie named Nor98, a genetic role was firstly described by Moum *et al.* (2005) at the codons 141 and 154, these are involved to different risk degree to occurrence the disease, in particular, an increased susceptibility to the atypical has been reported with AF<sub>141</sub>RQ allele (Moum *et al.*, 2005; Pongolini *et al.*, 2009). In Mediterranean countries such as Italy, Greece and Spain ARQ/ARQ is a very frequent genotype detected in animals sick with scrapie (Coseddu *et al.*, 2007; Nonno *et al.*, 2003). However, many authors reported ARQ-associated SNPs related to scrapie-resistance (Acutis *et al.*, 2004; Goldmann *et al.*, 2005; Meydan *et al.*, 2013; González *et al.*, 2014).

Sarda sheep breed shows two alleles associated to classical scrapie resistance, AT<sub>137</sub>RQ and ARQK<sub>176</sub> (Maestrone *et al.*, 2009; Vaccari *et al.*, 2009; Bucalossi *et al.*, 2011). In a clinical study that involved the Barbado breed, heterozygous ARQ-ARK<sub>171</sub> showed a prolonged average incubation time of 30 months before the onset of clinical signs (Greenlee *et al.*, 2012) and similarly, in infected Suffolk sheep, heterozygous AM<sub>112</sub>RQ-AT<sub>112</sub>RQ showed lower attack and increased survival rates (Chianini *et al.*, 2013).

The *Sopravissana* breed is known as *Popes' sheep* reared in the Apennines, in Central Italy. The breed is originated from a local population named Vissana crossed with Spanish Merino and Rambouillet in the 18th and early 19th century (Figure 1) (Lasagna *et al.*, 2011). *Sopravissana* rams are horned and the females are polled. The breed is adapted to the local

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Key words: Prion; *PRNP* gene; SNPs; Local breed.

Acknowledgements: the study was supported by the Italian Ministry of Health grant IZSUM 08/2011 RC (*Evaluation of new protective alleles for scrapie AT<sub>137</sub>RQ/ARQK<sub>176</sub> in ovine breeds in Umbro-Marchigiano territory, Italy*). The research was also financially supported by Fondazione Cassa di Risparmio di Perugia, project number 2013.0208.021. The authors want to thank the two anonymous referees for their valuable comments and constructive suggestions.

Received for publication: 4 September 2015.

Accepted for publication: 16 October 2015.

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Italian Journal of Animal Science 2015; 14:4251  
doi:10.4081/ijas.2015.4251

environment. It is a fine to medium woolled breed kept for milk and meat production (Bigi and Zanon, 2008). Together with the other Merino-derived Italian breed *Gentile di Puglia*, *Sopravissana* had a strong effect in the establishment of the technological, social and economic characteristics of the rural populations living in the Centre and South of Italy (Sarti *et al.*, 2006). In particular, the products of *Sopravissana* are high quality wool, *romanesco* cheese, and lambs named *abbacchio* (butchered at about 40 days of age); all these products have their own characteristics and can therefore be recognized for their real typicality (Sarti *et al.*, 2001). The *Sopravissana* have a population of 8500 heads (83 livestock farming) (<http://www.assonapa.it>). For this reason, a number of projects are being planned to save this breed from extinction, by monitoring the residual populations, setting up conservation flocks and applying new severe morphological standards. In this context, the European Union decided to financially support the *Sopravissana* by Italian regional funding.

The aim of this study was to assess, by molecular tools, the allelic and genotypic frequencies in *Sopravissana* *PRNP* gene. Moreover we evaluated *PRNP* polymorphisms

in ARQ/ARQ animals to identify the frequency of potential protective ARQ allele variants able to increase scrapie resistance.

## Materials and methods

### Samples collection

A total of 72 whole blood samples, from December 2012 to November 2013 with Vacutainer system, in tubes added with EDTA as anticoagulant were collected, and stored at -20°C until analyses. All the animals were females clinically healthy with a minimum age of 18 months, belonging to 10 flocks of *Sopravissana* breed in Marche and Lazio Regions. No outbreaks were involved in the study. Samples from rams were excluded, since it is not possible to find ARQ/ARQ rams in population because they are culled during the performance test.

### DNA extraction and genotyping analysis

Genomic DNA was isolated by a semiautomatic extractor BioSprint® 96 One-For-All Vet Kit, (Qiagen®, Hilden, Germany) in according to the manufacturer's protocol. Genotyping analysis of codons 136, 154, and 171 was performed following the protocol described by Vaccari *et al.* (2009), slightly modified: for the Allelic Discrimination Assay, 2.3 µL of genomic DNA were transferred into four different PCR mixtures (codon 136, codon 154, codon 171-1 and codon 171-2) containing 1X TaqMan® Universal PCR Master Mix, primers forward and reverse 900 nM each, and 150 nM of TaqMan® MGB probes (Table 1) to a final volume of 22 µL. Sample's genotypes were carried out with a Fast Real Time PCR StepOne Plus real-time PCR System (Applied Biosystems®, Foster City, CA, USA) with the follow thermic profile: 50°C (for 2 min), followed by 10' at 95°C, 15" at 95°C for and 1' at 62°C for 40 cycles. The results were analysed by SDS v.2.2.2 software.

PrP genotypes were reconstructed on the assumption that all polymorphisms are mutually exclusive. The allele shall be defined by reference to the amino acids (three-letter code *e.g.* ARQ or ARR) encoded by codons 136, 154 and 171 of the prion protein gene (*PRNP*).

Mutated alleles are indicated with the three-letter code plus the additional polymorphic amino acid and its position (*e.g.* AT<sub>112</sub>RQ) (Vaccari *et al.*, 2009).

**Table 1. Primers and probes sequences.**

Codons	Sequences
136-F	5'-CTGCAGCTGGAGCAGTGGTA-3'
136-R	5'-ATAGTAACGGTCTCATAGTCATTGC-3'
154-F	5'-TGGCAATGACTATGAGGACCG-3'
154-R	5'-TGGTCTGTAGTACACTTGGTTGGG-3'
171-F	5'-GTTACCCCAACCAAGTGTACTACAGA-3'
171-R	5'-TGTTGACACAGTCATGCACAAAAG-3'
Probe MGB 136 A	5'-FAM-TCATGGCACTTCC-3'
Probe MGB 136 V	5'-VIC-CTCATGACACTTCC-3'
Probe MGB 154 H	5'-FAM-ACTATCATCGTAAAAACA-3'
Probe MGB 154 R	5'-VIC-TACTATCATGAAAAATG-3'
Probe MGB 171 R	5'-FAM-CCAGTGGATCGGTATA-3'
Probe MGB 171 H	5'-VIC-ACCAGTGGATCATTAT-3'
Probe MGB 171 Q	5'-VIC-ACCAGTGGATCAGTATA-3'

MGB, minor groove binder.

### Sequencing analysis

The *PRNP* gene portion (1229 bp) where the entire CDS sequence is located, has been amplified in the homozygous ARQ samples as described by Vaccari *et al.* (2009) in a Mastercycler®ep gradient S (Eppendorf®, Hamburg, Germany). PCR fragments were purified with QIAquick® PCR purification kit (Qiagen®, Hilden, Germany) according to manufacturer's recommendations. Purified amplicons have been sequenced for both the strands. Sequencing reaction was carried out with primers T3 (5'-TTTACGTGGGCATTTGATGC-3') and T4 (5'-GGCTGCAGGTAGACTCC-3') (Vaccari *et al.*, 2009) using Big Dye Terminator Cycle sequencing Kit v1.1 (Applied Biosystems®) and detected with ABI PRISM 3100 apparatus (Applied Biosystems®). Individual chromatograms were read and sequences were aligned to the *Ovis sp.* gene for prion protein PrP, complete cds (Accession number, GenBank D38179.1) with ClustalW tool of BioEdit 7.0.9 software (Hall, 1999).

### Data analysis

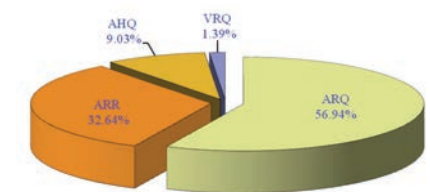
The allelic and genotypic frequencies were computed by Excel software. Deviation from Hardy-Weinberg equilibrium was evaluated through square test using R software (R Development Core Team, 2013).

## Results and discussion

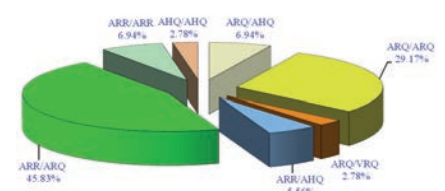
ARQ was the predominant allele, followed by the ARR, AHQ and VRQ alleles at lower frequencies (Figure 2). The frequencies of the



**Figure 1. Sopravissana sheep breed.**



**Figure 2. Allelic frequencies.**



**Figure 3. Genotypic frequencies.**

alleles observed in other researches on different Italian breeds, confirm our results: Pongolini *et al.* (2009) obtained similar results in 5 main Italian breeds (*Sarda*, *Bergamasca*, *Appenninica*, *Massese*, and *Comisana*); Vaccari *et al.* (2009) investigated same alleles in *Sarda* breeds including in their study the protective role of some new alleles against classical scrapie. Comparing our data with observations in other European countries during the past decade was noted that, the more common allele in Belgium (79.3%) and in Great Britain (52.3%) was ARR (Dobly *et al.*, 2013; Ortiz-Pelaez *et al.*, 2014); these values are quite higher than the ARR frequency observed in this study, probably due to the effect of different European Genetic Selection Plans against this disease.

The highest two genotypic frequencies were ARR/ARQ and ARQ/ARQ (Figure 3); the observed values were comparable to that reported by Vaccari *et al.* (2009) in the *Sarda* breed and to the ones reported by Pongolini *et al.* (2009) in other Italian breeds (*Appenninica*, *Comisana* and *Sarda* breeds). The ARR/ARQ genotype was the most frequent also in British sheep population (29.5%) (Baylis *et al.*, 2004). A Belgian sheep population resulted to have ARR/ARR as dominant genotype (Dobly *et al.*, 2013).

All genotypes were in Hardy-Weinberg equilibrium and no deviation was detected. A total of 21 ARQ/ARQ animals were found, thus analysis sequences were performed because in Italy this is a very frequent genotype and it is known that not all exposed ARQ/ARQ sheep develop scrapie (Nonno *et al.*, 2003; Laegreid *et al.*, 2008).

This study provides data about two non-synonymous (L141F, H143R) and two synonymous polymorphisms at codons 231 and 237. No novel amino acid substitution was found. L141F and H143R frequencies observed in *Sopravissana* were calculated (Table 2).

AF<sub>141</sub>RQ allele, responsible of susceptibility to atypical scrapie, was detected in the *Sopravissana*; such as in other Italian breeds: *Appenninica*, *Bergamasca*, *Biellese* and *Sarda* (Acín *et al.*, 2004; Maestrone *et al.*, 2009; Pongolini *et al.*, 2009; Vaccari *et al.*, 2009). AF<sub>141</sub>RQ allele was also found in Spanish sheep (Acín *et al.*, 2004), in Finnish sheep (Hautaniemi *et al.*, 2012) and in Chinese local sheep breeds (Lan *et al.*, 2014).

As reported in Table 2, *Sopravissana* sheep presented the AR<sub>143</sub>RQ allele in a similar frequency to that detected in Spanish sheep breeds (0-16.3%) by Acín *et al.* (2004), and also observed at lower level in Finnish Aland breed (1.0%) (Hautaniemi *et al.*, 2012) and in Italian

**Table 2. ARQ/ARQ frequencies of prion protein polymorphisms.**

	Codon	Sequence	Aminoacid	%
141	Wt	CTT	L	76.2
	Mut	TTT	F	23.8
143	Wt	CAT	H	83.3
	Mut	CGT	R	16.7
231	Wt	AGG	R	92.9
	Mut	CGG	R	7.1
237	Wt	CTG	L	92.9
	Mut	CTC	L	7.1

Wt, wild type; Mut, mutation.

*Biellese* breed (0.2%) (Acutis *et al.*, 2004). It has to be noted that, AR<sub>143</sub>QR allele was reported by Acín *et al.* (2004) in scrapie-affected animals, thus it might be considered to have not a protective role against classical scrapie.

## Conclusions

The results showed that the ARQ/ARQ sheep should be considered as a genetic class, which may potentially include animals with different scrapie susceptibility because of the presence of additional polymorphisms. This may allow the application of alternative strategies for breeding programmes, for instance in some rare breeds or in particular farms that show high frequency of the scrapie susceptible-associated ARQ allele. However, more evidence is required to support these findings before they can result in new scrapie resistance strategies in the ovine breeding programmes.

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