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**Prion protein gene polymorphisms in classical scrapie-affected flocks of sheep
in Central Macedonia**

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**Πολυμορφισμοί του γονιδίου της πρωτεΐνης prion σε θετικές,
ως προς την κλασσική τρομώδη νόσο,
εκτροφές προβάτων στην Κεντρική Μακεδονία**

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ABSTRACT. The allele and genotype frequency distributions of the prion protein gene polymorphisms at codons 136, 154 and 171 were determined by real-time PCR for 1,456 sheep from 7 classical scrapie-affected flocks of Thessaloniki and Imathia, Central Macedonia, Greece. The blood samples were collected by official veterinarians and were examined by the National Reference Laboratory (NRL) for TSEs, Veterinary Laboratory of Larisa, Greece, in the framework of the National Program for Scrapie Surveillance and Control between 2009 and 2013. Among the 1,456 sheep, 340 were of Chios breed, 633 Chios crossbred and 483 crossbred. The examined sheep showed high genotype variability, as a total of 7 haplotypes and 23 different genotypes were found. The predominant allele and the predominant genotype were ARQ and ARQ/ARQ respectively, in all breeds studied, followed by the ARR allele and the ARR/ARQ genotype. The TRQ allele was frequent in Chios and Chios crossbred, while the VRQ allele was rare for all the breeds. Interestingly, 3 genotypes (ARH/TRQ, ARR/ARK and ARK/VRQ) were detected for the first time in Greece and two of them (ARH/TRQ and ARK/VRQ) have, to our knowledge, never been previously reported. Furthermore, it is emphasized that our country outnumbers all European countries in classical scrapie cases of sheep every year. Therefore, there is an urgent need to reduce the incidence of classical scrapie through the implementation of selective breeding programs. This is supported by the fact that the prevalence of classical scrapie in the Greek sheep population is highly associated with the predominant genotype ARQ/ARQ. Therefore, the elimination of the ARQ/ARQ and the other susceptible genotypes (belonging to Risk Groups 3 and 5, according to the National Scrapie Plan of Great Britain) would reduce dramatically the incidence of classical scrapie in Greece.

Keywords: PrP gene polymorphisms, scrapie, Real-time PCR

ΠΕΡΙΛΗΨΗ. Για την έρευνα αυτή πραγματοποιήθηκε αιμοληψία σε 1.456 πρόβατα, ηλικίας 1-7 ετών, από 7 θετικές εκτροφές της Ημαθίας και της Θεσσαλονίκης, ως προς την κλασική τρομώδη νόσο, στα πλαίσια της εφαρμογής του Εθνικού Προγράμματος Επιτήρησης, Ελέγχου και Εξάλειψης των Μεταδοτικών Σπογγωδών Εγκεφαλοπαθειών (ΜΣΕ) στα μικρά μηρυκαστικά του Υπουργείου Αγροτικής Ανάπτυξης & Τροφίμων, για τα έτη 2009-2013. Τα αιμοδείγματα συλλέχθηκαν από κρατικούς κτηνιάτρους των κτηνιατρικών υπηρεσιών της Ημαθίας και Θεσσαλονίκης και οι γονοτυπικές εξετάσεις πραγματοποιήθηκαν στο Εθνικό Εργαστήριο Αναφοράς για τις ΜΣΕ, Κτηνιατρικό Εργαστήριο Λάρισας με τη μέθοδο της αλυσιδωτής αντίδρασης της πολυμεράσης σε πραγματικό χρόνο (Real-time PCR). Από τα 1.456 πρόβατα που εξετάστηκαν, τα 340 ήταν φυλής Χίου, τα 633 ημίαιμα Χίου και τα 483 ημίαιμα. Τα εξεταζόμενα ζώα παρουσίασαν μεγάλη γενετική ποικιλομορφία, καθώς βρέθηκαν 7 διαφορετικά αλληλόμορφα και 23 διαφορετικοί γονότυποι. Ο κυρίαρχος γονότυπος σε όλες τις φυλές ήταν ο ARQ/ARQ, ακολουθούμενος από τον ARR/ARQ. Ο απλότυπος TRQ ήταν συχνός στα Χιώτικα και στα ημίαιμα Χίου πρόβατα, ενώ ο απλότυπος VRQ ήταν σπάνιος σε όλες τις φυλές. Είναι αξιοσημείωτο ότι 3 γονότυποι (ARH/TRQ, ARR/ARK και ARK/VRQ) ανιχνεύθηκαν για πρώτη φορά στην Ελλάδα. Επιπλέον, 2 από αυτούς τους γονότυπους (ARH/TRQ, και ARK/VRQ) δεν έχουν, καθ' όσον γνωρίζουμε, αναφερθεί ποτέ μέχρι σήμερα. Επίσης, δίνεται έμφαση στο γεγονός ότι στη χώρα μας παρατηρούνται τα περισσότερα κρούσματα κλασικής τρομώδους νόσου των προβάτων κάθε χρόνο σε όλη την Ευρώπη. Συνεπώς είναι επιτακτική ανάγκη να μειωθεί η επίπτωση της νόσου στην Ελλάδα, μέσω της εφαρμογής προγραμμάτων επιλογής/αναπαραγωγής ανθεκτικών προβάτων (selective breeding programs) ως προς την κλασική τρομώδη νόσο. Σε αυτό συνηγορεί το γεγονός ότι ο επιπολασμός της νόσου στην Ελλάδα συνδέεται με τον γονότυπο ARQ/ARQ, ο οποίος είναι ο πιο συχνός γονότυπος. Άρα η δραστική μείωση του ARQ/ARQ και άλλων ευαίσθητων γονότυπων θα προκαλέσει δραματική μείωση της επίπτωσης της νόσου, προς όφελος της προβατοτροφίας και της εθνικής μας οικονομίας. .

Λέξεις-Κλειδιά: τρομώδης νόσος, γονοτυπική ανάλυση, πρωτεΐνη prion, αλυσιδωτή αντίδραση πολυμεράσης

INTRODUCTION

Scrapie is a fatal, progressive, neurodegenerative disease affecting sheep and goats. It has been known since 1732, when it was first described in the UK (McGowan 1922) and belongs to the family of Prion diseases or Transmissible Spongiform Encephalopathies (TSEs). The term “prion” was proposed by Prusiner in 1982, in order to denote a small proteinaceous infectious particle, which is resistant to inactivation by most procedures. According to the Prion Hypothesis, infectious prion particles are composed largely, if not entirely, of an abnormal isoform (PrP^{Sc}) of the prion protein, which is encoded by a chromosomal gene. (Prusiner 1991). The *PRNP* gene encodes the prion protein (PrP), which plays a major role in the disease process (Goldmann 2008, Houston et al., 2015). In the PrP genes of humans, mice and sheep, amino acid polymorphisms associated with disease susceptibility and pathogenesis have been found (Belt et al., 1995). In sheep, amino acid polymorphisms at many positions (89, 94, 101, 112, 127, 128, 132, 134, 135, 136, 137, 138, 141, 143, 145, 146, 149, 151, 152, 154, 157, 159, 160, 163, 164, 167, 168, 169, 171, 172, 174, 175, 176, 180, 183, 184, 185, 189, 193, 195, 196, 199, 211, 213, 220, 224, 241) have been described (Goldmann et al., 1990, 1991, 1994, 2005, Laplanche et al., 1993, Hunter et al., 1993, 1994, 1996, 1997, Ikeda et al., 1995, Belt et al., 1995, De Silva et al., 2003, Acutis et al., 2004, F. Guan et al., 2011, Alvarez et al., 2011, Oner et al., 2011, Zhao et al., 2012, Hautaniemi et al., 2012, Meydan et al., 2013, Curcio et al., 2015), but the polymorphisms at codons 136, 154 and 171 have been demonstrated to be of major importance, as they modulate the susceptibility/resistance of sheep for scrapie (Cloucard et al., 1995, Hunter et al., 1996, Smits et al., 1997). These polymorphisms are Alanine (A), Valine (V) or Threonine (T) at codon 136, Arginine (R) or Histidine (H) at codon 154 and Glutamine (Q), R, H or Lysine (K) at codon 171.

The five most common haplotypes are ARR, ARQ, AHQ, ARH and VRQ (Baylis et al., 2004, Dobby et al., 2013). Additional haplotypes (TRQ, ARK, VRR, AHR, VHQ and TRR) have been reported so far (Kutzer et al., 2002, De Silva et al., 2003, Acutis et al., 2004, Billinis et al., 2004, F. Guan et al., 2011, Psifidi et al., 2011, Oner et al., 2011, Alvarez et al., 2011,

Zhao et al., 2012, Meydan et al., 2012, Granato et al., 2013). According to the National Scrapie Plan for Great Britain, the fifteen most common genotypes have been classified into five risk groups (R1-R5), with R1 (ARR/ARR genotype sheep) indicating the lowest risk of scrapie and R5 (VRQ/VRQ, VRQ/ARQ and other VRQ-encoding genotypes) associated with the highest susceptibility (DEFRA, 2001). The disease is mainly associated with the ARQ/ARQ genotype (R3) in the sheep breeds (the so called “non-valine” or “alanine” breeds) where the VRQ allele is rare or absent, such as in many Mediterranean sheep breeds (Greek, Italian, Spanish), in Suffolk breed, in Canadian sheep etc (Hunter et al., 1997, Billinis et al., 2004, Ekateriniadou et al., 2007, Koutsoukou-Hartona et al., 2009, Harrington et al., 2010, Curcio et al., 2015).

A new form of scrapie was discovered in Norway in 1998 (named atypical scrapie or Nor98) (Benestad et al., 2003). It has been reported in several European countries (Fediaevsky et al., 2008), and the majority of sheep affected with atypical scrapie is between five and ten years of age or more, in contrast to the younger age (between three and five years) of the sheep affected with classical scrapie. It's noteworthy that only a single scrapie-positive per affected flock was identified in most cases (Luhken et al., 2007, Benestad et al., 2008). Recent evidence linking new variant Creutzfeldt-Jakob disease (nvCJD) in humans to BSE in cattle has increased attention for all TSEs, including scrapie (www.cabi.org). Scrapie (classical and atypical) is not considered a public health risk, but BSE has been linked to new variant Creutzfeldt-Jakob disease (Eloit et al., 2005, Spiropoulos et al., 2011).

Classical scrapie is a relatively common problem for Greek sheep farmers. Our country has the highest number of sheep scrapie cases in Europe every year. For example, in 2015 Greece reported 188 cases of classical scrapie in sheep, followed by Italy with 141, Romania with 98 and Spain with 69 cases, while all the other European countries presented only 50 cases in total (Boelaert et al., EFSA Journal 2016). Scrapie was firstly diagnosed in Greece in 1986 (Leontides et al., 2000), while nine positive flocks were found till 2001, as a result of the Passive Surveillance Program. From 2002, when the Active Surveillance Program

began, the positive flocks were increased year by year coming up to 223 (217 flocks with classical scrapie and 6 flocks with atypical scrapie) in 2008 (Koutsoukou et al., 2009). The total number of sheep classical scrapie cases in Greece during the period 2002-2015 was 5,448 (more than in any other European country), while only 30 atypical scrapie cases were also reported (Boelaert et al., EFSA Journal

The blood samples were collected by official veterinarians.

Among the 1,456 sheep, 340 were of Chios breed, 633 Chios crossbred and 483 crossbred (Table 1).

DNA Extraction, Amplification and Genotyping Analysis

All analyses were performed at the National Ref-

Table 1: Number of sheep sampled from each breed

Breed	Number of flocks	Number of sheep		Totals
		Rams	Ewes	
Chios	1	14	326	340
Chios Crossbred	4	30	603	633
Crossbred	2	17	466	483
Totals	7	61	1,395	1,456

2016). That's why it is imperative to combat classical scrapie in Greece, which can be achieved with proper implementation of selective breeding programs. The predominant genotype of the scrapie-affected sheep in Greece is ARQ/ARQ, at an approximately 70% percentage, followed by ARQ/AHQ, VRQ/VRQ and VRQ/ARQ (Billinis et al., 2004, Ekateriniadou et al., 2007a, Koutsoukou et al., 2009). Therefore, the elimination of these genotypes (belonging to Risk Groups 3 and 5, according to the National Scrapie Plan of Great Britain) with the successful implementation of selective breeding programs could reduce dramatically the incidence of classical scrapie in Greece.

The aim of this study was to present the allelic and genotype frequencies of 1,456 sheep from 7 scrapie-affected flocks in Central Macedonia (Thessaloniki and Imathia), sorted by their breed (Chios, Chios crossbred and Crossbred) from 2009 to 2013.

MATERIALS AND METHODS

Sheep

Blood samples were obtained from a total of 1,456 sheep (61 rams and 1,395 ewes), aged 1-7 years old, originating from 7 scrapie-affected flocks of Thessaloniki and Imathia (Central Macedonia, Greece), in the framework of the National Program for Scrapie Surveillance and Control, between 2009 and 2013.

erence Laboratory (NRL) for TSEs, Veterinary Laboratory of Larisa, Greece. The method used for scrapie genotype determination is based on multiplex real-time PCR technology.

DNA extraction

Sheep blood sample was collected using absorbing paper cards (Whatman FTA ELUTE Micro Cards). Blood spots (each of equal diameter per sample) were cut from the cards and the genomic DNA was extracted from circular spots using deionized water and direct heating using a thermoblock at 97 °C for 30min.

Real-time PCR melting curve genotyping

Genotyping was performed using the LightMix 480HT Scrapie Susceptibility Mutation Detection Kit (TIB MolBiol, Germany) and LightCycler® 480 Genotyping Master hot start reaction mix and probe melting-curve based genotyping kit (Roche Applied Science). The PCR reactions were conducted with the LightCycler® 480 II Real Time PCR thermal cycler (Roche Applied Science), according to the manufacturer's instructions. LightMix 480HT Scrapie Susceptibility Mutation Detection Kit uses 3 combinations of probes, each of them specific to codons 136, 154 and 171 respectively. In each real-time PCR reaction,

mutations within amplicons were detected during melting curve analysis, based on fluorescence resonance energy transfer (FRET) signal measurement. Melting peaks of PCR product–probe hybrids were obtained. Subsequently, melting curves peak analysis was performed to determine genotype at codons 136 [GCG (Ala-A), GTC (Val-V)], 154 [(CGT (Arg-R) and CAT (His-H)] and 171 [CAG (Gln-Q), CAT (His-H), CGT (Arg-R) and AAG (Lys-K)]. The melting peaks which were obtained by testing of samples were compared with the respective peaks of control samples, i.e. DNA extracts from the blood of sheep with known genotypes. These controls were purchased from VLA laboratories (UK) and were analyzed using the same protocol.

RESULTS

The PrP allelic frequencies of the 1,456 sampled sheep are summarized in Table 2. Seven alleles, of codons 136, 154 and 171 (ARR, ARQ, AHQ, ARH, VRQ, TRQ, ARK) were observed.

The most frequent allele was ARQ, with a mean frequency 62.74%. The second most frequent allele was ARR with 17.38%, followed by AHQ (8.41%), TRQ (6.05%) and ARH (3.57%). The ARK allele was found at an average frequency of 0.48%, while the VRQ allele was detected at low frequencies (1.37%) (Table 2).

The frequencies of PrP genotypes are presented in

Table 3. It is noteworthy that twenty three (23) different genotypes were identified in the sampled sheep. The fifteen (15) commonly reported PrP genotypes (included in the NSP) presented a total frequency of 87.91 %. Apart from them, eight (8) different genotypes were observed with a total frequency of 12.09%.

The ARQ/ARQ genotype, which is classified in the Risk Group R3, was the most frequent genotype (40.52%). The second most frequent genotype was ARR/ARQ (Risk Group R2) with 20.60 %, followed by ARQ/AHQ (Risk Group R3) with 10.10 % and ARQ/TRQ (which is not classified in any risk group by NSP) with 7.14 %. The genotype ARQ/TRQ was much more frequent in Chios breed and Chios crossbred (7.65 % and 10.58 % respectively) than in crossbred (2.28 %). The genotype ARH/ARQ (Risk Group R3) was found at frequency 4.33%, followed by the most resistant genotype ARR/ARR (Risk Group R1) with 3.85%, ARR/AHQ with 3.16 %, ARQ/VRQ (Risk Group R5) with 1.58%, ARR/TRQ with 1.51% and ARR/ARH with 1.17 %

NSP type III (Risk Group R3) was the most frequent (56.66 % of all animals), followed by type II (Risk Group R2) with 24.93%, type I (Risk Group R1) with 3.85%, type V (Risk Group R5) with 1.99% and type IV (Risk Group R4) with 0.48% in decreasing order of frequency. The remaining genotypes (12.09 %) were not classified in any NSP risk group (Table 3).

Table 2. Allelic frequencies of PrP polymorphisms at codons 136, 154 and 171

ALLELES	BREED							
	CHIOS		CHIOS CROSSBRED		CROSSBRED		TOTAL	
	n	%	n	%	n	%	n	%
ARR	68	10.00	190	15.01	248	25.67	506	17.38
ARQ	486	71.47	802	63.35	539	55.80	1,827	62.74
AHQ	39	5.74	97	7.66	109	11.28	245	8.41
ARH	37	5.44	31	2.45	36	3.73	104	3.57
VRQ	5	0.73	21	1.66	14	1.45	40	1.37
TRQ	42	6.18	115	9.08	19	1.97	176	6.05
ARK	3	0.44	10	0.79	1	0.10	14	0.48
TOTAL	680	100.00	1,266	100.00	966	100.00	2,912	100.00

Table 3. Genotype frequencies of PrP polymorphisms at codons 136, 154 and 171

NSPa	PrP GENOTYPE	BREED							
		CHIOS		CHIOS		CROSSBRED		TOTAL	
		n	%	n	%	n	%	n	%
Type I	ARR/ARR	4	1.18	17	2.69	35	7.25	56	3.85
Type II	ARR/ARQ	45	13.23	119	18.80	136	28.16	300	20.60
Type II	ARR/AHQ	7	2.06	12	1.90	27	5.59	46	3.16
Type II	ARR/ARH	4	1.18	4	0.63	9	1.86	17	1.17
									24.93
Type III	ARQ/ARQ	180	52.94	258	40.76	152	31.47	590	40.52
Type III	ARQ/AHQ	24	7.06	62	9.80	61	12.63	147	10.10
Type III	ARH/ARQ	25	7.35	21	3.32	17	3.52	63	4.33
Type III	AHQ/AHQ	2	0.59	5	0.79	5	1.04	12	0.82
Type III	AHQ/ARH	1	0.29	2	0.31	6	1.24	9	0.62
Type III	ARH/ARH	2	0.59	0	0.00	2	0.41	4	0.27
									56.66
Type IV	ARR/VRQ	1	0.29	4	0.63	2	0.41	7	0.48
Type V	ARQ/VRQ	4	1.18	10	1.58	9	1.86	23	1.58
Type V	VRQ/AHQ	0	0.00	1	0.16	3	0.62	4	0.27
Type V	VRQ/VRQ	0	0.00	2	0.31	0	0.00	2	0.14
Type V	ARH/VRQ	0	0.00	0	0.00	0	0.00	0	0.00
									1.99
n.c	ARQ/TRQ	26	7.65	67	10.58	11	2.28	104	7.14
n.c	ARR/TRQ	2	0.59	16	2.53	4	0.83	22	1.51
n.c	ARQ/ARK	2	0.59	7	1.11	1	0.21	10	0.69
n.c	AHQ/TRQ	3	0.88	10	1.58	2	0.41	15	1.03
n.c	TRQ/TRQ	4	1.18	9	1.42	1	0.21	14	0.96
n.c	ARH/TRQ **	3	0.88	4	0.63	0	0.00	7	0.48
n.c	ARR/ARK *	1	0.29	1	0.16	0	0.00	2	0.14
n.c	ARK/VRQ **	0	0.00	2	0.31	0	0.00	2	0.14
									12.09
	TOTAL	340	100.00	633	100.00	483	100.00	1,456	100.00

^a Risk group classification, according to the National Scrapie Plan (NSP) of Great Britain

n.c Not classified in any risk group by NSP

* Genotype detected for the first time in Greece

** The 2 genotypes have, to our knowledge, never been previously reported

DISCUSSION

The haplotype with the highest frequency was ARQ, that was found at an average frequency of 62.74 % for all breeds. The frequency of the allele ARQ in Chios breed and in Chios crossbred was 71.47% and 63.35% respectively. These high percentages are in agreement with the previously reported results for Chios breed (Ekateriniadou et al., 2007b, Psifidi et al., 2011, Ekateriniadou et al., 2007a). Higher frequencies of the ARQ allele have been reported in scrapie-affected sheep (Ekateriniadou et al., 2007a). Similar frequencies of the ARQ allele were reported in Italy (Granato et al., 2013, Martemucci et al., 2015), in Brazil (Ianella et al., 2012) and in Turkey (Meydan et al., 2012), whereas higher frequencies (74-86%) were observed in Awassi and Assaf sheep populations (Gootwine et al., 2008).

The ARQ/ARQ genotype was the predominant genotype with a mean of 40.52%. The frequency in Chios breed was 52.94% and that of Chios crossbred was 40.76%. Compared with the results of our study, same frequencies were reported in Canada which concerned 1990 healthy sheep from 3 scrapie-affected flocks (ARQ allele 62.2%, ARQ/ARQ 38.4%, Harrington et al., 2010). It is noteworthy that the frequency of the ARQ allele has significantly decreased since then, as a result of selection for resistant PrP genotypes in Ontario sheep (frequency of the ARQ haplotype 28% in 2012) and in sheep from other provinces of Canada from 2005 to 2012 (Cameron et al., 2014).

Furthermore, the ARQ allele and the ARQ/ARQ genotype were found to be the predominant allele and genotype respectively, in Finland (Hautaniemi et al., 2012), in China (Zhao et al., 2012), in Brazil (Ianella et al., 2012), in Turkey (Alvarez et al., 2011, Meydan et al., 2012), in East Asian sheep (Tsunoda et al., 2010) and in Pakistan (Babar et al., 2009) among other countries. Sheep with the genotype ARQ/ARQ are highly susceptible to scrapie, while this is the most common genotype among scrapie-affected sheep of Greek positive flocks with 68.35% in Chios crossbred, 70.39% in other crossbred (Ekateriniadou et al., 2007a), 72.73% (88 ARQ/ARQ from 121 scrapie-affected sheep) in crossbred (Billinis et al., 2004) and 75.58% (Koutsoukou-Hartona et al.,

2009). Therefore, if the frequency of the ARQ/ARQ genotype is reduced through the selective breeding program, the incidence of classical scrapie in Greece will be dramatically reduced.

The second most frequent genotype was ARQ/ARR with a mean frequency of 20.60% for all breeds and 13.23% in Chios breed, which is similar to the 11.35% (Psifidi et al., 2011) and 14.50% (Billinis et al., 2004).

The most resistant genotype ARR/ARR was found with a low mean proportion 3.85% in the study population and 1.18% in Chios breed, which is in agreement with previous studies of Greek sheep breeds (Koutsoukou-Hartona et al., 2009, Ekateriniadou et al., 2007a, Ekateriniadou et al., 2007b, Psifidi et al., 2011 and Billinis et al., 2004). High frequencies of the ARR/ARR genotype were reported in the UK, France, the Netherlands, Belgium, Cyprus, Germany, Hungary, Poland, Canada and other countries where selective breeding programs have been implemented. For example, 77.14% was reported in Berrichon du Cher in Poland (Grochowska et al., 2014), 82.4% in Cyprus (Boelaert et al., EFSA Journal 2016), whereas the frequencies of the ARR allele in rams of French breeds ranged from 68% to 100% (Palhiere et al., 2008).

The genotype ARQ/AHQ was found with a mean proportion of 10.10%, while Billinis et al. found 17.6% in crossbred sheep from scrapie-affected flocks and 12.40% in crossbred scrapie-affected sheep and Ekateriniadou et al., found 24.51% in Chios crossbred scrapie-affected sheep. Therefore, the genotype ARQ/AHQ is often observed in sheep positive for classical scrapie and belongs to the susceptible genotypes. As for the VRQ allele, the frequency in the study holdings was 1.37% (0.73% in Chios breed), supporting previous studies in Chios breed, in which low (0.40%, Psifidi et al., 2011), or null (Ekateriniadou et al., 2007a, b, Billinis et al., 2004) frequencies of the VRQ allele were observed. Among classical scrapie-affected sheep in Greece, the total frequency of the VRQ-related genotypes (belonging to Risk Groups NSP-4 + NSP-5) was 7.63% for all Greek sheep breeds (Koutsoukou-Hartona et al., 2009), 0.63% for Chios crossbred and 17.32% for other crossbred sheep, especially Karagouniko breed (Ekateriniadou et al., 2007a). So the

VRQ-related genotypes, mainly ARQ/VRQ and less the VRQ/VRQ and the others, are sometimes observed in scrapie-affected sheep in Greece. The VRQ-related genotypes were responsible for the 202/257 (78.6%) of the positive cases for classical scrapie in the Netherlands (Hagenaars et al., 2010). In addition, in the UK the greatest scrapie risk by far was for the VRQ-encoding genotypes ARQ/VRQ, ARH/VRQ and VRQ/VRQ (Baylis et al., 2004). On the other hand, the VRQ allele was absent in 16 Chinese local sheep breeds, as all sheep were homozygous for A at codon 136 (AA₁₃₆) (Lan et al., 2014).

The five haplotypes described above and the fifteen PrP genotypes, which are pairings of these five alleles defined by the polymorphisms at codons 136, 154 and 171, were included in the National Scrapie Plan for Great Britain (DEFRA, 2001). Apart from them, two additional alleles and eight additional PrP genotypes were found in our study. The sheep included in the present study showed a high degree of genetic variability, which can be possibly attributed to the absence of scrapie genotype-based selection of animals. In all countries where selective breeding programs have been implemented, genetic variability was decreased. For example, in Canada 12 genotypes of sheep were observed in 2005 in Ontario sheep, whereas only 6 genotypes were observed in 2012 after 7 years of selection for resistant genotypes (Cameron et al., 2014). Also, in Belgium many genotypes (from Risk Groups R3 and R5) disappeared, as a result of the selection program from 2006 to 2011 (Dobly et al., 2013).

The mean frequency of the genotype ARQ/TRQ was 7.14% (7.65% in Chios breed and 10.58% in Chios crossbred), which is consistent with previous reports in Chios breed (10.00%, Billinis et al., 2004, 11.94%, Psifidi et al., 2011). This genotype has also been observed in Chinese Hu sheep, (9.44%, F. Guan et al., 2011), in Northwestern China (0.53%, Zhao et al., 2012) and in Turkey (4.03%, Oner et al., 2011 & 6.0%, Alvarez et al., 2011). The rare genotype TRQ/ARH was found only in Chios crossbred and in Chios breed and the frequencies were 0.63% and 0.88% respectively, at a mean of 0.48%. To our knowledge, it's the first time that this genotype is detected. The mean frequency of the rare genotype ARR/ARK was 0.14% (0.29% in Chios breed, 0.16%

in Chios crossbred). This genotype is detected for the first time in Greece. Till now, it has been reported in Chinese Hu sheep (0.56%, F. Guan et al., 2011), in North Western China (0.40%, Zhao et al., 2012), in Turkey (0.1%, Meydan et al., 2012) and in Italy (1.7% in Foza breed, Granato et al., 2013). The very rare genotype ARK/VRQ was found only in Chios crossbred (0.31%) and the average frequency in the study population was 0.14%. To our knowledge, it's the first time that it is detected.

To sum up, the dominant genotype (ARQ/ARQ with 40.52%) in the study sheep population is at the same time, by far, the most common genotype of the scrapie-affected sheep in Greece. Furthermore, the ARQ/AHQ (10.10%, third genotype in decreasing order of frequency) is also often observed in scrapie-affected sheep (Billinis et al., 2004, Ekateriniadou et al., 2007a). These 2 genotypes account for more than 50% of the study sheep population. The frequency of these genotypes, as well as of all the genotypes belonging to Risk Groups 3,4 and 5, could be significantly decreased as a result of the implementation of selective breeding programs, resulting in lower incidence of classical scrapie in the future. Experience with the successful implementation of selective breeding programs in the European countries (UK, France, Cyprus, the Netherlands, Belgium) has shown an impressive reduction in the incidence of the disease (Dawson et al., 2008, Palhiere et al., 2008, Hagenaars et al., 2010, Nodelijk et al., 2011, Ortiz-Pelaez and Bianchini, 2011, Dobly et al., 2013). For example, 3,063 classical scrapie cases in sheep were reported in Cyprus in the period 2002-2015. The 96.3% of them was reported until 2009 and only 3.7% in the period 2010-2015. The same happened in the UK and France (Boelaert et al., EFSA Journal 2016). This reduction in the incidence of classical scrapie in Cyprus, the UK and France is due to the beneficial effects of the selective breeding programs implemented in the 2000s.

CONCLUSIONS

The examined sheep from 7 scrapie-affected flocks of Central Macedonia showed high genotype variability, as a total of 7 haplotypes and 23 different genotypes were detected at codons 136, 154 and 171. Interestingly, three genotypes were detected for the first time in Greece, while two of them (ARH/TRQ

and ARK/VRQ) have, to our knowledge, never been previously reported.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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