Short communication

Identification of genetic variation in the major histocompatibility complex gene region in Turkish sheep breeds

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(Received 14 March 2016; Accepted 7 September 2016; Published 31 October 2016)

ABSTRACT

The major histocompatibility complex (MHC) in sheep, Ovar-Mhc, remains poorly characterized relative to other domestic animals. However, its basic structure is similar to that of other mammals, comprising class I, II and III regions. In this study, the Ovine MHC class II *DRB1* and *DRB3* genes were amplified by polymerase chain reaction in eight sheep breeds reared in Turkey. Informative restriction fragment length polymorphisms (RFLPs) were obtained with five restriction enzymes for *DRB1* and with two restriction enzymes for *DRB3*. The digestion of *DRB1* exon 2 with *Nci*I, *Sac*I, *Sac*II, *Hin*1I each produced three genotypes and two alleles (viz., a and b) with frequencies of 0.69 and 0.31; 0.65 and 0.35; 0.91 and 0.09; 0.57 and 0.43, respectively. The digestion of *DRB1* exon 2 with *Dde*I produced four genotypes and three alleles (viz., a, b and c) with frequencies of 0.62, 0.28 and 0.10, respectively. On the other hand, the digestion of *DRB3* exon 2 with *Nde*II and *Bsa*I each produced three genotypes and two alleles (viz., a and b) with frequencies of 0.72 and 0.28; 0.96 and 0.04, respectively. This study presents the genetic profiles of the exon 2 region of the MHC *DRB1* and *DRB3* genes in native Turkish sheep breeds.

Keywords: *DRB1*, *DRB3*, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP)
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The major histocompatibility complex is a large genomic region or gene family that is found in most vertebrates, which encodes MHC molecules. MHC molecules play an important role in the immune system and auto-immunity (Tizard, 2004). The MHC was discovered during tissue transplantation studies in mice and was first characterized for its role in histocompatibility. Subsequently, its roles in immune regulation and several other functions were discovered. The primary function of the MHC is to code for specialized antigen-presenting receptor glycoproteins, known as histocompatibility molecules or MHC molecules (Dukkipati *et al*., 2006). The major histocompatibility complex in sheep is poorly characterized when compared with other domestic animals. However, its basic structure is similar to that of mammalian MHC molecules, comprising class I, II and III regions. The MHC genes of sheep are known as ovar and are located on chromosome 20. Ovar class II genes encode polymorphic glycoproteins composed of heterodimers of noncovalently linked alpha (α) and beta (β) subunits, which play a pivotal role in the initiation of the immune response to pathogen-derived peptide antigens (Brujeni *et al*., 2009). The schematic structure of the ovine MHC is illustrated in Figure 1.

Class I MHC glycoproteins are expressed on the surface of all nucleated somatic cells. They are found in highest concentration on lymphocytes and macrophages, and consist of heterodimers of and/or heavy chain noncovalently linked to a light β2-microglobulin chain. Class I genes include eight exons and seven introns. The *DRB* locus is highly polymorphic among the class II MHC genes (Anderson & Rask, 1988). This locus encodes heterodimeric peptide-binding proteins and proteins that modulate antigen loading onto the MHC class II. Relative to other parts of the MHC, the class III region has the highest gene density, with the least number of pseudogenes (Kulski *et al*., 2002). However, some of the genes located in this region are not involved in immune system functions. The MHC class III region encodes other immune components including the complement system (e.g., C2, C4 or factor B) and cytokines (e.g., TNF-α).

RL: http://www.sasas.co.za
ISSN 0375-1589 (print), ISSN 2221-4062 (online)
Publisher: South African Society for Animal Science
http://dx.doi.org/10.4314/sajas.v46i4.4

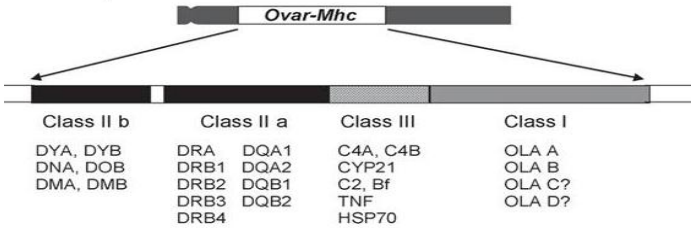

Figure 1 Schematic presentation of the structure of Ovar-Mhc (The major histocompatibility complex) (Dukkupati *et al*., 2006)

Studies of genetic variation in Ovar-Mhc class II genes have shown that the expressed *DRB* locus is highly polymorphic (Schwaiger & Epplen, 1995; Schwaiger *et al*., 1996; Jugo & Vicario, 2000; Konnai *et al*., 2003; Ballingall *et al*., 2008; Nikbakht *et al*., 2012; Lotfi *et al*., 2012; Shen *et al*., 2014; Takeshima *et al*., 2014). In particular, a high polymorphism level is present in exon 2, which encodes the antigen-binding site (Escayg *et al*., 1997; Konnai *et al*., 2003). There is little knowledge of MHC polymorphism in Turkish sheep. Only one study has been performed by Bozkaya & Kurar (2005) until today. Therefore, in this study, the ovine MHC, class II *DRB1* and *DRB3* genes were analysed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) in eight Turkish sheep breeds.

Genetic variation was analysed in sheep from eight breeds reared in Turkey, including the White Karaman, Daglıc, Awassi, Sakiz, Kivircik, Karayaka, Malya and Morkaraman (15 to 25 sheep per breed) (Table 1).

Table1 Source of samples used for RFLP analyses

Breeds	Source
White Karaman	Konya
Daglic	Afyon
Awassi	Urfa
Sakiz	Balikesir
Kivircik	Bursa
Karayaka	Tokat
Malya	Konya
Morkaraman	Kars

Blood samples were obtained from Ankara University Faculty of Agriculture. Genomic DNA was isolated from blood samples according to the method describe in Miller *et al*. (1988).

In this study, genetic variability in the ovine MHC, class II *DRB1* and *DRB3* genes were analysed using the PCR-RFLP technique.

OLA-DRB1 was amplified according to Konnai *et al*. (2003). Nested PCR was used to amplify the second exon of the *DRB1* gene. The first round of PCR was performed with primers OLA-ERB1 (5′-ccggaattcccgtctctgcagcacatttctt-3′) and HL031 (5′-tttaaattcgcgctcacctcgccgct-3′). The PCR reactions were carried out in a total volume of 25 µl containing the following reaction mixture: 2 µl of 10× reaction buffer with KCl, 1.5 mM MgCl2, 1.2 mM of dNTP mix, 20 µM of each primer, 2.5 U of Taq polymerase and 20 ng of purified sheep DNA. The following amplification profile was used: initial denaturation at 94 °C for 5 min, 15 cycles at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. The second round of PCR amplification was carried out using 5 µl of the resultant mixture, with the addition of primers OLA-ERB1 and OLA-XRB1 (5′-gctcgagcgctgcacagtgaaactc-3′). The thermal cycle included an initial denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. The amplified PCR products were electrophoresed on 1% agarose gels to verify the fragment sizes. The

amplified second exon of the *DRB1* gene was digested with five restriction enzymes to verify the expected recognition sites. The informative restriction enzymes used for the *DRB1* PCR fragments were *Nci*I, *Sac*I, *Sac*II *Hin1*I and *Dde*I.

OLA-DRB3 was amplified according to Amills *et al*. (1996). PCR reactions were performed using primers DRB1.1 (5′-tatcccgtctctgcagcacatttc-3′) and DRB1.2 (5′-tcgccgctgcacactgaaactctc-3′). Amplification was performed in a 25-µl reaction volume containing 2.5 µl of 10× reaction buffer with KCl, 1.5 mM MgCl2, 1.0 mM of dNTP mix, 10 µM of each primer, 1.5 U of Taq polymerase and 20 ng of purified sheep DNA. The thermal cycle was programmed for an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. The amplified *DRB3* PCR products were digested with two restriction enzymes *Nde*II and *Bsa*I.

The informative restriction enzymes were analysed using 15 to 25 individuals from each population. The digested fragments were separated electrophoretically on 2 or 3% agarose gels in 1× TBE buffer, stained with ethidium bromide and photographed using a Vilber Lourmat gel imaging system.

Gene and genotypic frequencies were estimated by direct counting. Deviations from Hardy-Weinberg (HW) equilibrium were estimated by chi-square (χ^2) (Duzgunes *et al*., 1983).

In the present study, polymorphism in exon 2 of the *Ovar-DRB1 and Ovar-DRB3* loci was analysed by PCR-RFLP of DNA samples obtained from eight Turkish sheep breeds. The average size of the PCR-amplified *Ovar-DRB1* region observed by agarose gel electrophoresis for all the populations studied was 296 bp. Five restriction enzymes generated informative digestion profiles in *DRB1* exon 2. The *Nci*I, *Sac*I, *Sac*II, *Hin*1I and *Dde*I restriction enzymes generated different digestion profiles of DNA samples from eight Turkish sheep breeds (Table 1). The average size of the PCR-amplified *Ovar-DRB3* region for all the populations studied was 285 bp. The *Nde*II and *Bsa*I restriction enzymes generated informative digestion profiles in *Ovar-DRB3* exon 2 (Table 2).

Table 2 Restriction fragment patterns generated from analysis of *Ovar-DRB1 and Ovar-DRB3* exon 2 gene segments after endonuclease digestion of DNA samples from Turkish sheep breeds

Restriction enzyme	Patterns observed (bp)
*Nci*I	a:296 b:150,146
*Sac*I	a:296 b:208,88
*Sac*II	a:296 b:229,67
*Hin*1I	a:296 b:178,118
*Dde*I	a:296 b:181,115 c:40, 256
*Nde*II	a:285 b:200,85
*Bsa*I	a:285 b:182,103

The enzymatic digestion of exon 2 of *DRB1* with *Nci*I, *Sac*I, *Sac*II, *Hin*1I each generated three genotypes and two alleles, and *Dde*I digestion generated four genotypes (aa, bb, ab and ac) and three alleles. The enzymatic digestion of exon 2 of *DRB3* with *Nde*II and *Bsa*I each generated three genotypes and two alleles.

Allele a was obtained from the *Nci*I, *Sac*I, *Sac*II and *Dde*I digestions of *DRB1* exon 2 and was found to be more frequent in all of these sheep breeds. On the other hand, allele b obtained from the digestion of *DRB1* with *Hin1*I was more frequent in the Kivircik and White Karaman breeds (Table 3). The *DRB1* exon 2 showed high heterozygosity by digestion with *Hin1*I. The lowest heterozygosity was obtained by digestion of *DRB1* exon 2 with *Sac*II and *Nci*I. The HW test showed that the studied most of the breeds fit the theoretical proportions for the *Nci*I, *Sac*I, *Sac*II and *Dde*I digestions of exon 2 of *DRB1* (Table 4) (P <0.05). However, White Karaman, Sakiz, Karayaka and Malya don't fit the theoretical proportions for the *Hin*1I digestions of this gene (Table 4) (P <0.05).

Table 3 Allelic frequencies of various patterns in exon 2 of the *DRB1* gene after restriction endonuclease digestion of DNA samples from Turkish sheep breeds.

Breeds	Allelic frequency										
	*Nci*I		*Sac*I		*Sac*II		*Hin*1I		*Dde*I		
	a	b	a	b	a	b	a	b	a	b	c
White Karaman	0.89	0.11	0.68	0.32	0.89	0.11	0.33	0.68	0.55	0.45	0.00
Daglic	0.72	0.28	0.61	0.39	0.72	0.28	0.55	0.45	0.57	0.39	0.04
Awassi	0.88	0.12	0.68	0.33	0.88	0.12	0.61	0.39	0.58	0.31	0.11
Sakiz	1.00	0.00	0.70	0.30	1.00	0.00	0.63	0.37	0.71	0.25	0.04
Kivircik	0.92	0.08	0.75	0.25	0.92	0.08	0.45	0.55	0.80	0.20	0.00
Karayaka	0.93	0.08	0.65	0.35	0.93	0.08	0.65	0.35	0.60	0.00	0.40
Malya	1.00	0.00	0.53	0.47	1.00	0.00	0.75	0.25	0.53	0.29	0.18
Morkaraman	0.93	0.08	0.67	0.33	0.93	0.08	0.58	0.42	0.69	0.22	0.09

Table 4 Chi-square (χ^2) values for Hardy-Weinberg equilibrium

Breeds	Chi-square (χ^2) value						
	*Nci*I	*Sac*I	*Sac*II	*Hin*1I	*Dde*I	*Nde*II	*Bsa*I
White Karaman	3.63	3.49	2.38	5.56*	0.00	2.14	0.00
Daglic	0.15	0.19	3.67	3.33	5.323	2.20	0.00
Awassi	3.66	1. 86	2.34	3.56	7.780*	16.17*	6.65*
Sakiz	7.21*	0.16	0.00	7.38*	4.069	17.16*	5.65*
Kivircik	1.13	3.06	3.28	0.67	0.00	0.59	2.37
Karayaka	0.28	0.24	3.38	6.00*	0.00	0.16	0.00
Malya	5.24*	0.17	0.00	3.86*	18.889*	2.48	0.00
Morkaraman	0.14	0.94	3.38	0.34	1.344	4.27*	0.00

*P <0.05

The digestion of *DRB3* exon 2 with *Nde*II and *Bsa*I each resulted in three genotypes and two alleles. The *Nde*II- and *Bsa*I-generated allele a of exon 2 of *DRB3* was more frequent in all of the studied sheep breeds (Table 5). The *DRB3* gene had a high heterozygosity for *Nde*II digestion of exon 2. The HW test showed that the studied most of the breeds fit the theoretical proportions for the *Nde*II and *Bsa*I digestions of the exon 2 region of *DRB3* (Table 4) (P <0.05). Table 6 shows the allele frequencies of various patterns in the exon 2 of *DRB1 and DRB3* after RFLP analysis.

This study presents the first insights and RFLP profiles of the *DRB1*and *DRB3* genes in native sheep breeds in Turkey. Alleles found in this study were similar to those previously identified and reported by Konnai *et al*. (2003) and Amills *et al*. (1996). Although novel alleles were not identified, further detailed DNA

sequence analysis of the exon 2 regions of *Ovar-DRB1* and *Ovar-DRB3* from Turkish sheep breeds will be carried out.

Table 5 Allelic frequencies of various patterns in the *DRB3* gene after restriction endonuclease digestion of DNA samples from Turkish sheep breeds

Breeds	Allelic frequency			
	*Nde*II		*Bsa*I	
	a	b	a	b
White Karaman	0.87	0.13	1.00	0.00
Daglic	0.83	0.18	1.00	0.00
Awassi	0.50	0.50	0.95	0.05
Sakiz	0.50	0.50	0.94	0.06
Kivircik	0.75	0.25	0.81	0.19
Karayaka	0.61	0.39	1.00	0.00
Malya	0.90	0.10	1.00	0.00
Morkaraman	0.73	0.27	1.00	0.00

Table 6 Allelic frequencies of various patterns in exon 2 of the *DRB1 and DRB3* genes after RFLP analysis

	a	**b**	**c**
*Nci*I	0.69	0.31	-
*Sac*I	0.65	0.35	-
*Sac*II	0.91	0.09	-
*Hin1*I	0.57	0.43	-
*Dde*I	0.62	0.28	0.10
NdeI	0.72	0.28	-
BsaI	0.96	0.04	-

Several methods have been employed for typing *Ovar-DRB1* genes in various sheep breeds and have revealed extensive polymorphism at these loci. Among these methods, PCR-RFLP analysis has been suggested for the effective typing of *DRB1* alleles of farm animals (Amills *et al*. 1996; Konnai *et al*. 2003; Dongxiao & Yuan 2004; Gruszczynska *et al*. 2005; Brujeni *et al*. 2009). To date, 106 *Ovar-DRB1* alleles have been identified by DNA sequencing of exon 2 from various breeds of sheep (Schwaiger *et al*., 1995; Schwaiger *et al*., 1996; Jugo & Vicario, 2000; Konnai *et al*., 2003; Ballingall *et al*., 2008). The results of the studies have shown that MHC polymorphism in animals correlates with immunity and the immune response.

The *Nde*II b allele has been correlated with the occurrence of Ile 37, 67 substitutions. The positions are expected to be involved in the formation of one region of the antigen recognition site (ARS). The resolving power of this method allows the detection of amino acid substitutions at the ARS of the DR molecule (a MHC class II cell surface receptor) may help to understand the genetic basis of disease resistance (Amills *et al*. 1996).

There is little knowledge of MHC polymorphism in ruminants compared with that of human beings and mice. Therefore, similar studies should be extended to ruminants, including the analysis of additional MHC

regions. Allelic variation in the MHC region and its association with various traits in immunity has yet to be analysed in ruminants. The current study is a preliminary analysis of the polymorphism of the MHC *DRB1* and MHC *DRB3* genes in Turkish sheep breeds, which provides a basis for further studies. Knowledge of MHC polymorphism in Turkish sheep breeds allows the development of sheep by helping to understand the genetic basis of their resistance to disease. A larger sample size and additional molecular analysis are required to verify these results and determine the association between the MHC *DRB* genes and disease resistance in ruminants.

Acknowledgments

This study was performed by Fatma Ilhan in partial fulfilment of the Ph.D. degree in Biometry and Genetics, Animal Science Dept., Selcuk University. This project was supported by a grant from The Scientific Research Project Coordinating Office of Selcuk University. (Project No: 10101036).

Authors' Contributions

Conception, design, data collection, analyses and drafting of paper - FI; Critical revision – IK; Data collection, final approval of version to be published – AT.

Conflict of Interest Declaration

There are no conflicts of interest.

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