Exon 2 *Ovar-DRB1* gene polymorphism in the Iranian Sangsari sheep

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Abstract

The major histocompatibility complex (MHC) plays a central role in the control of disease resistance and immune response. Extensive genetic diversity in MHC genes provides a valuable source for genetic improvement, via selection, in many domestic animals. Exon 2 of the class II MHC, termed *Ovar-DRB1* in domestic sheep (*Ovis aries*), has been suggested as important disease resistance and immune response gene. We characterized *Ovar-DRB1* in DNA samples from 138 individuals of a population of the Iranian Sangsari sheep breed using PCR-RFLP. Eight *DRB1* alleles were identified among Iranian Sangsari sheep, including one previously unrecognized allele. Eight homozygous genotypes were observed: *a, b, c, d, f, g, h* and N. Genotype *bb* was the most common pattern (46 of 138). Heterozygous genotypes (*ag, cb, cd, bf, and bN) were also observed*. The observed homozygosity and heterozygosity values were 0.6377 and 0.3623, respectively, vs expected values of 0.220 and 0.779. Iranian Sangsari population deviate significantly from the theoretical proportions (FIS = 0.5283; p = 0.0005). In conclusion, PCR-RFLP analysis allows rapid identification of *Ovar-DRB1* types and discrimination of homozygous and heterozygous genotypes. This study indicates that the exon 2 region of the *Ovar-DRB1* gene is highly polymorphic in the Iranian Sangsari sheep breed.

Introduction

In sheep, the major histocompatibility complex (MHC) class II genes, termed Ovar, are located on chromosome 20 and encode polymorphic glycoproteins composed of nine covalently linked α and β subunits. Among the Ovar MHC class II genes, the expressed *DRB1* locus is highly polymorphic (Ballingall *et al*., 1992; Konnai *et al*., 2003b; Fabb *et al*., 2004), particularly in exon 2, which encodes the antigen-binding site (Outteridge *et al*., 1996; Konnai *et al*., 2003b). Polymorphism of *DRB1* enables the gene to recognize a variety of foreign peptides to trigger immune reactions (Tizard, 2004). In all species where the MHC loci have been analyzed, maps of highly polymorphic sites have been used to identify the genetic factors associated with disease resistance and susceptibility within this region, and perform comparative genomic studies. Furthermore, such maps contribute to an understanding of the evolution of MHC genes in different species, to the development of broadly effective vaccines, and to plan breeding strategies aimed at improving resistance to infectious diseases.

In sheep, the *Ovar-DRB1* polymorphism has been defined using several PCR-based methods including sequence-specific oligonucleotide probe analysis (Schwaiger *et al*., 1993, 1994), single-strand conformational polymorphism (SSCP, Kostia *et al*., 1998; Jugo and Vicario, 2000) and PCR-restriction fragment length polymorphism (RFLP) analysis (Dutia *et al*., 1994; Rasool *et al*., 2000; Dongxiao and Yuan, 2004; Gruszczynska *et al*., 2004).

This study aimed to analyze the genetic diversity of the *Ovar-DRB1* exon 2 locus in the Iranian Sangsari breed of sheep using PCR-RFLP.

Materials and Methods

Sampling and DNA extraction

A total of 138 blood samples were collected from a population of Sangsari sheep located in the Semnan province of Iran. Whole blood was preserved in acid citrate dextrose solution and stored at -20°C. DNA was isolated from whole blood as described by Sambrook

et al. (1989) with some modifications. Briefly, 300 µl aliquots of whole blood were added to 500 µl red blood cell lysis buffer (20 mM Tris-HCl, pH 7.6), mixed, incubated at room temperature for 10 min and centrifuged at 12000 g for 20 sec. The pellet was resuspended in 600 µl of cell lysis buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA, 0.1% sodium dodecyl sulfate), homogenized and 300 µl denaturation solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5 % N- lauroylsarcosine) was added, incubated at room temperature for 10 min, then 300 µl potassium acetate (3 M) was added, mixed for 30 sec, centrifuged for 3 min at 12000 g and the supernatant was transferred to a fresh tube containing 600 µl isopropanol to precipitate the DNA. The pellet was washed with 70% ethanol and resuspended in 50 µl TE (pH 7.6).

DRB1.2 amplification and RFLP analysis

The second exon (308 bp) of the *DRB1* gene was amplified by PCR in a final volume of 25 μL containing template DNA, 20 pmol of each primer (5'-ATCCTCTCTCTGCAGCACATTTCC -3' and 5'-TTTAAATTCGCGCTCACCTCGCCGCT -3'), PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl) with 1 mM $MgCl_2$, 0.25 mM dNTPs, and 1 U *Taq* DNA polymerase. PCR was performed at 94°C for 4 min, 35 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 1 min, followed by 72°C for 5 min. Contamination and self-priming controls were included in each round of PCR and 5 μL was electrophoresed on 1.5% agarose gels to check the quality and specificity of DNA fragment amplification. The restriction enzyme *Rsa*I was used to examine the nucleotide sequence variability at the *DRB1.2* locus. Aliquots (10 μL) of PCR product (308 bp) were digested with *Rsa*I according to the manufacturer's instructions (Fermentas, St. Leon-Rot, Germany), separated on 12% polyacrylamide gels in TBE, using *Msp*I-digested *pBR322* (Fermentas, Germany) as a molecular marker. After ethidium bromide staining, gels were photographed under UV light with a Gel Doc system (Vilber Lourmat Inc. Cedex, France) and the relative migration of the DNA bands was estimated.

Population genetic analysis

Gene and genotypic frequencies were estimated by direct counting. The Popgene 1.32 program (Yeh, 1997) was used to perform statistical analysis. Expected homozygosity and heterozygosity were determined using the Levene method (Levene, 1949). Deviations from Hardy–Weinberg (HW) equilibrium were estimated via the *F*IS parameter (Weir and Cockerham, 1984). Gene diversity was measured by the number of alleles (*n*a) and the unbiased expected heterozygosity (*h*e), according to the formula of Nei *et al*. (1973). The Ewens–Watterson neutrality test was performed using the method described by Manly (Manly, 1985).

Results

Polymorphism in the second exon of *DRB1 in* Iranian Sangsari sheep was analyzed using PCR amplification and RFLP. PCR amplification resulted in DNA bands of the expected size (308 bp) in PAGE (Figure 1). When the amplified products were cleaved using the restriction enzyme *Rsa*I, we observed 13 different digest patterns in the population of Iranian Sangsari sheep (Table 1). Seven of the *Rsa*I digest patterns were similar to those previously reported by Konnai *et al*. (2003a), including *RsaI a, b, c, d, f, g* and *h* (Figure 1). In addition, one putative novel *DRB1* PCR-RFLP *Rsa*I pattern (N), which has not been previously reported, was observed (Figure 1). The numbers of bands in the different restriction patterns ranged from 2 to 8, and the sizes of the bands observed in the different *Rsa*I patterns are illustrated in Table 1.

The numbers of animals demonstrating each of different patterns is summarized in Table 2. Allelic frequencies were determined by direct counting of each *RsaI* restriction pattern. Eight homozygous genotypes were observed: *a, b, c, d, f, g, h* and N (Figure 1). Genotype N (0.061) constituted 6.1% of total occurrences and *b* (0.398) constituted 39.8%. Genotype *bb* was the most common pattern (46 of 138). The frequency distribution of the patterns are shown in Table 2. Heterozygous genotypes (*ag, cb, cd, bf, and bN) were also observed*. The observed homozygosity and heterozygosity values were 0.6377 and 0.3623, respectively, vs. expected values of 0.220 and 0.779. The HW test indicated that the *RsaI* patterns observed deviate significantly from the theoretical proportions of the study population (*F*IS = 0.5336; $p = 0.0005$).

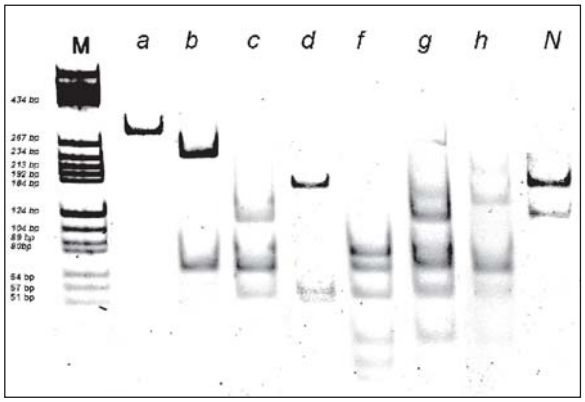

Figure 1: PCR-RFLP analysis of exon 2 of the *Ovar-DRB1* gene in Sangsari sheep. The 308 bp PCR-amplified DNA fragments were cleaved using *Rsa*I. The a-h and N genotypes are indicated. M: DNA fragment size marker *(Msp*I digest of *pBR*322).

Table 1: Genotypic frequencies of the second exon of the MHC *DRB* gene in Sangsari sheep.

Genotype		Allele	
*Rsa*I	*aa*: 308 bp	*Rsa*I	*a*: 308 bp
*Rsa*I	*bb*: 240 bp/68 bp	*Rsa*I	*b*: 240 bp/68 bp
*Rsa*I	*cc*: 117 bp/69 bp/68 bp /54 bp	*Rsa*I	*c*: 117 bp/69 bp/68 bp /54 bp
*Rsa*I	*dd*: 186 bp/68 bp/54 bp	*Rsa*I	*d*: 186 bp/68 bp/54 bp
*Rsa*I	*ff*: 84 bp/68 bp/54 bp/39 bp/33 bp/30 bp	*Rsa*I	*f*: 84 bp/68 bp/54 bp/39 bp/33 bp/30 bp
*Rsa*I	*gg*: 117 bp/68 bp/54 bp/39 bp/30 bp	*Rsa*I	*g*: 117 bp/68 bp/54 bp/39 bp/30 bp
*Rsa*I	*hh*: 147 bp/68 bp/54 bp/39 bp	*Rsa*I	*h*: 147 bp/68 bp/54 bp/39 bp
*Rsa*I	*NN*: 186 bp/122 bp	*Rsa*I	*N*: 186 bp/122 bp
*Rsa*I	*ag*: 308 bp /117 bp/68 bp/54 bp/39 bp/30 bp		
*Rsa*I	*bc*: 240 bp/117 bp/69 bp/68 bp /54 bp		
*Rsa*I	*bf*: 240 bp/84 bp/68 bp/54 bp/39 bp/33 bp/30 bp		
*Rsa*I	*bN*: 240 bp/186 bp/122 bp/68 bp		
*Rsa*I	*cd*: 186 bp/117 bp/69 bp/68 bp/54 bp		

Table 2: Genotypic and allelic frequencies of the second exon of the MHC *DRB* gene in Sangsari sheep.

Genotype	*n*	Frequency	Allele	Frequency
*Rsa*I *aa*	8	0.057	*Rsa*I *a*	0.072
*Rsa*I *bb*	46	0.333	*Rsa*I *b*	0.398
*Rsa*I *cc*	1	0.007	*Rsa*I *c*	0.141
*Rsa*I *dd*	1	0.007	*Rsa*I *d*	0.108
*Rsa*I *ff*	9	0.065	*Rsa*I *f*	0.079
*Rsa*I *gg*	16	0.115	*Rsa*I *g*	0.130
*Rsa*I *hh*	1	0.007	*Rsa*I *h*	0.007
*Rsa*I *NN*	6	0.043	*Rsa*I *N*	0.061
*Rsa*I *ag*	4	0.028		
*Rsa*I *bc*	9	0.065		
*Rsa*I *bf*	4	0.028		
*Rsa*I *bN*	5	0.036		
*Rsa*I *cd*	28	0.202		

Discussion

Sangsari sheep is an Iranian light weighted breed which is proposed to supply meat. Compared to cattle and other ruminant species, the *Ovar-DRB1* locus is poorly characterized, and the types and extent of polymorphism in this species have not been reported in sheep. Different methods have been employed to type *Ovar-DRB1* genes in various sheep breeds, revealing extensive polymorphism, and PCR-RFLP analysis has been suggested for typing *DRB1* alleles in farm animals (Amills *et al*., 1996; Gruszczynska *et al*., 2004). In the current study, polymorphisms in second exon of the *DRB* locus was investigated in Iranian Sangsari sheep. PCR-RFLP demonstrated the existence of 13 *DRB* types in the second exon of the Sangsari sheep population tested. A number of frequent *RsaI* patterns were observed, including *a, b, c, d, f, g, and h*, which correlate with the patterns in recent PCR-RFLP studies of *Ovar-DRB1* exon 2 polymorphism (Konnai *et al*., 2003b; Gruszczynska *et al*., 2004). Pattern *e*, reported by Konnai *et al*. (2003a) was not observed in our population of Sangsari sheep; however, we observed a new *Rsa*I restriction pattern (pattern N). This pattern had been previously reported by Gruszczynska *et al*. (2004) and introduced as restriction pattern 7. In this study of Sangsari sheep, *b* was the most frequent (0.398) exon 2 *RsaI* digestion pattern; however, in other breeds, including Polish Sheep, the most frequently observed pattern was *g* (~ 0.40, Gruszczynska *et al*., 2004). The HW test indicated that the studied Iranian Sangsari population deviates significantly from the theoretical proportions (*F*IS =0.5283; $p = 0.0005$). The single origin of the founder population of Sangsari sheep in Iran may account for this observation, and the limited variation may lead to insufficient maintenance of diversity, through a selection mechanism. Inbreeding and frequency dependent selection may be responsible for inadequate maintenance of MHC diversity in Sangsari sheep.

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