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 in domestic sheep (Ovar-DRB1), has been suggested as important disease resistance and immune response gene. We characterized 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: $a\bar{a}$, $b\bar{b}$, $c\bar{c}$, $d\bar{d}$, and $N\bar{N}$. Genotype $a\bar{a}$ was the most common pattern (46 of 138). Heterozygous genotypes 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 ($I_{S} = 0.5283$; $\phi = 0.0005$). In conclusion, PCR-RFLP analysis allows rapid identification of types and discrimination of homozygous and heterozygous genotypes. This study indicates that the exon 2 region of the 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 $\alpha$ and $\beta$ 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 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′-ATCCTCCTCTGCACGCACATTTC -3’ and 5′-TTTTATTGCTGCTACCTGCCGCTGCT -3’), PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl) with 1 mM MgCl₂, 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 Rsal was used to examine the nucleotide sequence variability at the DRB1.2 locus. Aliquots (10 μL) of PCR product (308 bp) were digested with Rsal according to the manufacturer’s instructions (Fermentas, St. Leon-Rot, Germany), separated on 12% polyacrylamide gels in TBE, using MspI-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 FIS parameter (Weir and Cockerham, 1984). Gene diversity was measured by the number of alleles (na) and the unbiased expected heterozygosity (he), 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 Rsal, we observed 13 different digest patterns in the population of Iranian Sangsari sheep (Table 1). Seven of the Rsal digest patterns were similar to those previously reported by Konnai et al. (2003a), including Rsal a, b, c, d, f, g and h (Figure 1). In addition, one putative novel DRB1 PCR-RFLP Rsal 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 Rsal patterns are illustrated in Table 1.

The numbers of animals demonstrating each of the different patterns is summarized in Table 2. Allelic frequencies were determined by direct counting of each Rsal 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, ch, 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 Rsal patterns observed deviate significantly from the theoretical proportions of the study population (FIS = 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 Rsal. The a-h and N genotypes are indicated. M: DNA fragment size marker (MspI digest of pBR322).](image-url)
new RsaI 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 (FIS = 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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