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Association of molecular polymorphism in exon 2 of *Ovar-DRB1* gene with weight traits in Iranian Makuie sheep breed

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Abstract: Exon 2 of the *Ovar-DRB1* gene of 90 Makuie sheep was amplified and analyzed by polymerase chain reaction and restriction fragment length polymorphism techniques. Ten alleles and 18 genotypes were identified. The frequencies of the genotypes were 0.317, 0.1585, 0.0121, 0.0365, 0.0121, 0.0243, 0.0243, 0.1097, 0.0121, 0.0487, 0.0121, 0.0365, 0.0365, 0.0487, 0.0121, 0.0121, 0.0487, and 0.0365 for AA, AB, AE, FF, AM, BO, EO, IO, OM, AP, BP, OP, PP, AQ, OQ, PQ, QQ, and AV, respectively. Allele frequencies were 0.4756, 0.0976, 0.0183, 0.0366, 0.0549, 0.0122, 0.1098, 0.0915, 0.0854, and 0.0183 for A, B, E, F, I, M, O, P, Q, and V, respectively. There was no significant ($P \geq 0.05$) deviation from Hardy–Weinberg equilibrium for this locus in Makuie sheep. Relationships between molecular polymorphism in the exon 2 region of *Ovar-DRB1* and weight traits were investigated. The results showed that the AB genotype was associated with the average of daily weight gain at 9 months and 12 months ($P \leq 0.05$). No correlation of exon 2 *Ovar-DRB1* variants with birth weight and other studied traits was detected. We expect that this gene can act as a candidate gene for genetic improvement of some weight traits in sheep breeding programs.

Key words: *Ovar-DRB1* gene, weight traits, Makuie sheep, polymorphism

1. Introduction

The Makuie sheep is as an Iranian native breed distributed in the cold, highland mountainous areas of Iran, especially in West Azerbaijan Province (1). The total population is estimated at approximately 2.7 million (2). They are mainly white-colored with black spots on the face and feet (3). They are primarily raised for meat and wool production. The produced wool is coarse and usually used for carpet weaving (4).

The major histocompatibility complex (MHC) plays a key role in immune response and has significant effects on reproductive traits in all mammals (5). This complex is generally found in almost all vertebrates. MHC molecules are glycoproteins on cell surfaces and present antigen activity-triggering T lymphocytes (6). MHC is classified into 3 classes (I, II, and III) according to cellular distribution, molecular weight, and function. MHC class I is found on all nucleated cells and presents antigen activity to CD^+8 T lymphocytes (7). MHC class II is found on the surface of antigen-presenting cells including B lymphocytes, macrophages, and dendritic cells, and they present antigen activity to CD^+4 T lymphocytes (6). The class II region is divided into 2 subregions, which are separated by at least 15 cM (8). MHC class III encodes the component of the complement system (9).

The ovine MHC was first identified about 27 years ago by using serological tests on lymphocyte antigens of sheep (10). The MHC of sheep is known as Ovar ('Ovar' representing *Ovis aries*) and is located on chromosome 20. The Ovar class II genes encode polymorphic glycoproteins composed of 9 covalently linked subunits (α and β subunits) that play a pivotal role in the initiation of the immune response against pathogen-derived peptide antigens (11). Among Ovar class II genes, the expressed DRB1 locus has been found to be highly polymorphic, and most of the polymorphic sites on DRB1 are located in exon 2, which encode the outer domain of MHC, especially the β-chain, which is the binding site for an antigen (11,12).

Several polymerase chain reaction (PCR)-based methods have been used for studying the polymorphism in

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MHC in sheep, including sequence-specific oligonucleotide probe analysis (13), single-strand conformation polymorphism (SSCP) (14,15), cloning and sequencing (16), PCR-restriction fragment length polymorphism (RFLP) (11,17), and direct sequence analysis of PCR products (18). To date, more than 160 *Ovar-DRB1* alleles have been identified by DNA sequencing of exon 2 from various sheep breeds (19). High polymorphism in the exon 2 sequence associates with immune response toward a wide variety of pathogens. Two MHC microsatellite alleles, GT/GA, were identified on the second intron of the *Ovar-DRB1* gene, closely linked to the exon 2 sequence and associated with production of the highest levels of antibodies in response to nematode parasites (20). These alleles are also expected to play an important role in the fertility of sheep (21). Moreover, in other studies a positive association was found between polymorphism in *Ovar-DRB1* and development of tumors produced by the bovine leukemia virus, suggesting that the differences in immune responses might be triggered by differences in MHC class II alleles (16). In addition to the central role of the MHC in host defense, MHC genotypes have been observed to be associated with production, reproduction, and growth traits in domestic animals (9,22,23).

In sheep, and especially Iranian native sheep breeds, our knowledge concerning the influence of the *Ovar-DRB1* gene polymorphism on weight gain, reproduction, and growth traits is still limit. The aims of the present work were to study polymorphism in exon 2 of the *Ovar-DRB1* gene by using PCR-RFLP and to investigate their association with weight traits in the Iranian Makuie sheep breed.

2. Materials and methods

2.1. Collecting weight trait data and blood samples

Ninety Makuie sheep of both sexes were randomly selected and their weight trait data were obtained based on the national sheep recording system from the Makuie Sheep Breeding Center. Blood samples from all selected sheep were collected. The whole blood was preserved in ethylenediamine tetra acetic acid (EDTA)-coated tubes and stored at –20 °C.

2.2. DNA extraction and amplification of exon 2 of the *Ovar-DRB1* gene

Genomic DNA was extracted from 0.3 mL of blood using a genomic DNA purification kit (Fermentas Cat. No. 0512) according to manufacturer's instructions. Quality and quantity of extracted DNA was measured on 0.8% agarose gel prepared in 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0), visualized with ethidium bromide (1.0 mg mL^{-1}), and photographed under UV light using a Gel-Doc image analysis system (Gel Logic 212 PRO, USA). The amplification of the exon 2 region of the *MHC-DRB1* gene was achieved using primers MHC-Forward (5´-TCTCTGCAGCACATTTCCTGG-3´) and MHC-Reverse (5´-CTCGCCGCTGCACAGTGAAAC-3´) targeting a fragment of 279 bp as described by Ammer et al. (20). The PCRs were performed in a final volume of 25 µL containing 100 ng of template DNA, 0.5 µL of each primer, 2.5 µL of 10X PCR buffer, 4 µL of 1.25 mM dNTP (BioFluxbiotech, http://biofluxbiotech.com), 1 µL of 50 mM $MgCl_2$ (CinnaGen, Tehran, Iran), and 0.5 µL of Tag DNA polymerase (CinnaGen) using a 96-well Eppendorf Mastercycler Gradient (Type 5331, Eppendorf AG, Hamburg, Germany). The solution was initially denatured at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 90 s, annealing at 60 °C for 30 s, and extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. Five microliters of PCR products were electrophoresed on 2% agarose gels in order to check the quality and specificity of DNA fragment amplification.

2.3. Polymorphism detection of *Ovar-DRB1* gene using RFLP

The restriction enzyme RsaI was used to examine the nucleotide sequence variability at the exon 2 region of the *MHC-DRB1* gene. Seven microliters of PCR product was treated with 0.5 U of RsaI, 1 µL of green buffer, and 6.5 µL of distilled water in a final reaction volume of 15 µL according to the manufacturer's instructions (Fermentas). The restricted fragments were analyzed by electrophoresis on 2% agarose gel in 0.5X TBE buffer, stained with 1.0 µg mL^{-1} ethidium bromide, and photographed under UV light using a Gel-Doc image analysis system (Gel Logic 212 PRO). The 100-bp ladder was used as the molecular size marker.

2.4. Statistical analysis

The allelic and genotypic frequencies and observed and expected Nei's heterozygosities ($H_E = 1 - \Sigma P^2_i$, where P_i is the frequency of allele i) were estimated by using PopGene32 version 1.31 (24). Hardy–Weinberg equilibrium testing was performed in PopGene32. Data on growth traits were collected based on the national sheep recording system. Breeding values were calculated based on a fixed-effects model using the DFREML procedure (derivative-free restricted maximum likelihood) (25):

$$Y_{ijklm} = \mu + YR_i + SX_j + BT_k + AD_l + AN_m + E_{ijklm},$$

where Y_{ijklm} is the dependent variable (birth weight or weight gain) evaluated on the *i*th level of years as a random factor (YR_i, i = 1, 2, 3,…, 21), the *j*th level of sex as a fixed factor (SX_j, j = 1 and 2), the *k*th level of offspring number in each birth as a fixed factor (BT_K, k = 1, 2, and 3), the *l*th level of mother's age as a fixed factor (AD_l, l = 1, 2,…,7), and the *m*th level of the random additive genetic effect (AN_m, m = number of animals for each trait). The variable

μ is the overall mean for each trait, and E_{ijklm} is the random error effect. SAS 9.1 was used to calculate least squares means and to make multiple comparisons among the various genotypes in the Makuie breed.

3. Results

3.1. PCR amplification of exon 2 of *Ovar-DRB1* gene and RFLP analysis

The exon 2 regions of the *Ovar-DRB1* gene (a fragment of 279 bp in length) were successfully amplified in our first attempt by the specific primers as described by Ammer et al. (20). All extracted DNAs from sheep blood samples yielded a specific single-band PCR product without any nonspecific bands. The 279-bp PCR products were digested with the restriction endonuclease RsaI. Ten alleles (A, B, E, F, I, M, O, P, Q, and V) and 18 genotypes (AA, AB, AE, FF, AM, BO, EO, IO, OM, AP, BP, OP, PP, AQ, OQ, PQ, QQ, and AV) were identified in the restriction pattern of exon 2 of the *MHC-DRB1* gene of the Makuie sheep breed (Figure; Table 1). The frequencies of the observed

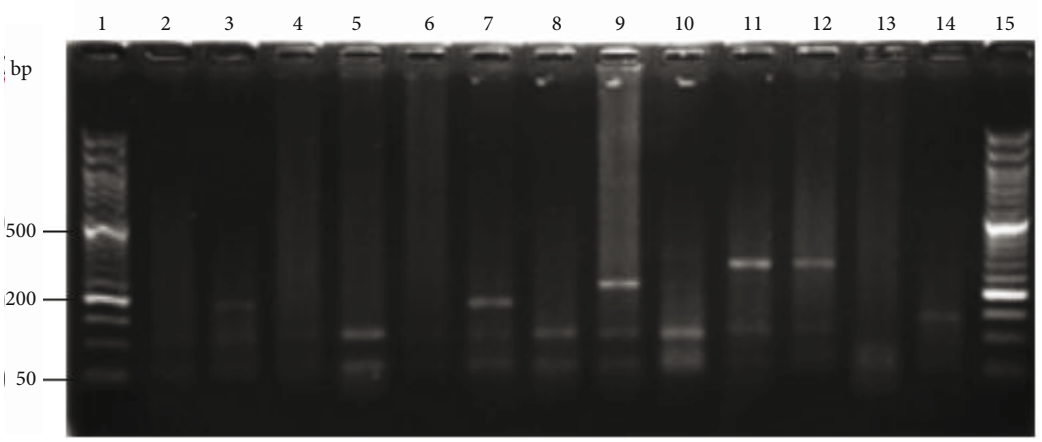

Figure. PCR-RFLP analysis of exon 2 of the *Ovar-DRB1* gene (279 bp) of Iranian Makuie sheep. Lanes 1 and 15: 50-bp ladder marker (Vivantis, Malaysia); lanes 2, 4, 5, 6, 8, and 10: genotype AA; lanes 3 and 7: genotype AB; lane 9: genotype AQ; lanes 11 and 12: genotype AP; lane 13: genotype AE; lane 14: genotype FF.

Table 1. Genotypes and alleles of exon 2 of *MHC-DRB1* gene in Makuie sheep breed.

Genotype	Allele
RsaI AA: 105 bp / 69 bp / 54 bp / 51 bp	RsaI A: 105 bp / 69 bp / 54 bp / 51 bp
RsaI AB: 123 bp / 105 bp / 69 bp / 54 bp / 51 bp	RsaI B: 123 bp / 105 bp / 51 bp
RsaI AE: 105 bp / 72 bp / 69 bp / 58 bp / 54 bp / 51 bp / 44 bp	RsaI E: 72 bp / 58 bp / 54 bp / 51 bp / 44 bp
RsaI FF: 135 bp / 54 bp / 51 bp / 39 bp	RsaI F: 135 bp / 54 bp / 51 bp / 39 bp
RsaI AM: 135 bp / 105 bp / 72 bp / 69 bp / 54 bp / 51 bp / 42 bp / 30 bp	RsaI I: 103 bp / 72 bp / 54 bp / 50 bp
RsaI BO: 240 bp / 123 bp / 105 bp / 51 bp / 39 bp	RsaI M: 135 bp / 72 bp / 42 bp / 30 bp
RsaI EO: 240 bp / 72 bp / 58 bp / 54 bp / 51 bp / 44 bp / 39 bp	RsaI O: 240 bp / 39 bp
RsaI IO: 240 bp / 103 bp / 72 bp / 54 bp / 50 bp / 39 bp	RsaI P: 279 bp
RsaI OM: 240 bp / 135 bp / 72 bp / 42 bp / 39 bp / 30 bp	RsaI Q: 228 bp / 51 bp
RsaI AP: 279 bp / 105 bp / 69 bp / 54 bp / 51 bp	RsaI V: 156 bp / 72 bp / 51 bp
RsaI BP: 279 bp / 123 bp / 105 bp / 51 bp	
RsaI OP: 279 bp / 240 bp / 39 bp	
RsaI PP: 279 bp	
RsaI AQ: 228 bp / 105 bp / 69 bp / 54 bp / 51 bp	
RsaI OQ: 228 bp / 240 bp / 51 bp / 39 bp	
RsaI PQ: 279 bp / 228 bp / 51 bp	
RsaI QQ: 228 bp / 51 bp	
RsaI AV: 156 bp / 105 bp / 72 bp / 69 bp / 54 bp / 51 bp	

genotypes were 0.317, 0.1585, 0.0121, 0.0365, 0.0121, 0.0243, 0.0243, 0.1097, 0.0121, 0.0487, 0.0121, 0.0365, 0.0365, 0.0487, 0.0121, 0.0121, 0.0487, and 0.0365 for AA, AB, AE, FF, AM, BO, EO, IO, OM, AP, BP, OP, PP, AQ, OQ, PQ, QQ, and AV, respectively. Allele frequencies were 0.4756, 0.0976, 0.0183, 0.0366, 0.0549, 0.0122, 0.1098, 0.0915, 0.0854, and 0.0183 for A, B, E, F, I, M, O, P, Q, and V, respectively (Table 2). The observed heterozygosity value was 0.5610. The expected heterozygosity value was 0.7359. The effective number of alleles (N_E) was 3.7231 (26) and Shannon's information index (I) was 1.7323 (27). The chi-square test showed no significant ($P \geq 0.05$) deviation from Hardy–Weinberg equilibrium for the studied locus in the studied population.

3.2. Association of exon 2 of *Ovar-DRB1* gene polymorphism with weight traits

A general linear mixed model revealed that the AB genotype was associated with weight at 9 months and weight at 12 months in the Makuie sheep breed, but no association was found between other observed genotypes and weight traits. The effects of the AA, AB, AE, FF, AM, BO, EO, IO, OM, AP, BP, OP, PP, AQ, OQ, PQ, QQ, and AV genotypes on birth weight, weaning weight, and weight at 6 month were not significant (Table 3).

4. Discussion

Different methods have been used for studying polymorphism of the *Ovar-DRB1* gene in various sheep breeds. The results revealed extensive polymorphism at this locus. In Finnish and Russian breeds, by using the SSCP technique, 7 new sequences and 19 alleles in exon 2 of 31 animals were detected (14). In the Latxa breed, 9 alleles were identified. Among the identified alleles, 8 alleles were similar to previously published alleles and 1, namely DRB* 14, was a new allele (15). Allele DRB1* 0403 and DRB1* 07012, identified in the Columbia, Polypay, and Rambouillet breeds, were positively correlated with lower OPP provirus levels. In Kazakh sheep, by using the RFLP technique, 14 alleles and 28 genotypes were detected (28). That study compared the frequency of genotypes

Table 2. Genetic diversity of *MHC-DRB1* locus in Iranian Makuie sheep breed.

Genetic diversity statistics	Value	Allele frequencies	Value	Genotypic frequencies	Number of sheep	Value
N_A	10.00	A	0.4756	AA	25	0.317
N_E	3.7231	B	0.0976	AB	13	0.1585
I	1.7323	E	0.0183	AE	1	0.0121
Observed homozygosity	0.439	F	0.0366	FF	3	0.0365
Observed heterozygosity	0.561	I	0.0549	AM	1	0.0121
Expected homozygosity	0.2641	M	0.0122	BO	2	0.0243
Expected heterozygosity	0.7359	O	0.1098	EO	2	0.0243
Average heterozygosity	0.7314	P	0.0915	IO	9	0.1097
		Q	0.0854	OM	1	0.0121
		V	0.0183	AP	4	0.0487
				BP	1	0.0121
				OP	3	0.0365
				PP	2	0.0365
				AQ	4	0.0487
				OQ	1	0.0121
				PQ	1	0.0121
				QQ	3	0.0487
				AV	2	0.0365

N_A = observed number of alleles. N_E = effective number of alleles. I = Shannon's information index.

Table 3. Effect of the *MHC-DRB1* gene genotype on growth traits in Iranian Makuie sheep breed.

Genotype	Trait				
	Birth weight	Weaning weight	Weight at 6 months	Weight at 9 months	Weight at 12 months
	NS	NS	NS	0.05	0.05
AA	0.025 ± 0.0013	0.07 ± 0.0021	0.13 ± 0.031	0.324 ± 0.021	0.418 ± 0.11
AB	0.043 ± 0.0211	0.12 ± 0.0032	0.16 ± 0.025	0.491 ± 0.014[a]	0.81 ± 0.06[a]
AQ	0.029 ± 0.0031	0.09 ± 0.0051	0. 11 ± 0.041	0.235 ± 0.03	0.51 ± 0.56
AP	0.03 ± 0.0061	0.086 ± 0.0041	0.14 ± 0.003	0.184 ± 0.026	0.53 ± 0.041
EO	0.019 ± 0.0012	0.072 ± 0.0035	0.132 ± 0.024	0.301 ± 0.04	0.40 ± 0.03
FF	0.023 ± 0.0022	0.11 ± 0.0052	0.09 ± 0.016	0.264 ± 0.018	0.61 ± 0.026
AE	0.043 ± 0.0041	0.09 ± 0.0036	0.098 ± 0.024	0.2917 ± 0.017	0.48 ± 0.03
IO	0.038 ± 0.0011	0.083 ± 0.0045	0.12 ± 0.008	0.199 ± 0.003	0.55 ± 0.019
PQ	0.028 ± 0.0091	0.078 ± 0.004	0.15 ± 0.032	0.328 ± 0.025	0.42 ± 0.035
OP	0.034 ± 0.0071	0.082 ± 0.003	0.096 ± 0.009	0.264 ± 0.019	0.56 ± 0.041
BO	0.040 ± 0.0062	0.075 ± 0.005	0.152 ± 0.0095	0.310 ± 0.018	0.58 ± 0.02
OM	0.032 ± 0.0082	0.094 ± 0.0029	0.124 ± 0.043	0.384 ± 0.05	0.63 ± 0.033
OQ	0.033 ± 0.0031	0.091 ± 0.0037	0.131 ± 0.035	0.201 ± 0.048	0.57 ± 0.06
PP	0.039 ± 0.0043	0.081 ± 0.0046	0.112 ± 0.04	0.362 ± 0.038	0.39 ± 0.018
AM	0.037 ± 0.0032	0.083 ± 0.005	0.095 ± 0.0085	0.331 ± 0.033	0.46 ± 0.026
QQ	0.026 ± 0.0035	0.084 ± 0.0039	0.115 ± 0.029	0.401 ± 0.029	0.43 ± 0.029
BP	0.028 ± 0.0062	0.079 ± 0.0043	0.089 ± 0.036	0.299 ± 0.024	0.40 ± 0.062
AV	0.035 ± 0.0015	0.08 ± 0.0035	0. 215 ± 0.035	0.340 ± 0.034	0.54 ± 0.017

NS: not significant. a: significantly different from other values in the same columns.

in hydatidosis-infected sheep with those of the control group. Their results showed that there was a positive and significant correlation between identified genotypes and resistance to hydatidosis (28).

In the present study, variation in the exon 2 sequence of the DRB1 locus was studied in Iranian Makuie sheep by PCR-RFLP analysis. Ten alleles (A, B, E, F, I, M, O, P, Q, and V) and 18 genotypes (AA, AB, AE, FF, AM, BO, EO, IO, OM, AP, BP, OP, PP, AQ, OQ, PQ, QQ, and AV) were observed in the exon 2 region of the *Ovar-DRB1* gene. Allele A and genotype AA were the most frequent, with the frequencies of 0.475 and 0.317, respectively. The results were partly in concordance with the findings of 2 previous studies (7,11). Allele RsaI Q was similar to pattern 6 in Polish Heath and Polish Lowland sheep and to allele b in Suffolk sheep. Allele RsaI P was similar to pattern 13 in Polish Heath and Polish Lowland sheep. Allele RsaI E was similar to allele f in Suffolk sheep and allele RsaI B was similar to allele d in Suffolk sheep. Other alleles, A, F, I, M, O, and V, were all new and were not found in other sheep breeds. Any differences between results reported herein and others may be due to difference in numbers of samples and species of sheep breeds. The chi-square test showed no significant ($P \geq 0.05$) deviation from Hardy–Weinberg equilibrium in the exon 2 of the *Ovar-DRB1* gene in the studied population.

In an earlier study, Nikbakht et al. (29) investigated polymorphism in exon 2 of the *Ovar-DRB1* gene in 3 Iranian sheep breeds including Lori-Bakhtiyari, Shal, and Zandi (fat-tailed sheep). In comparison with these other sheep breeds, the restriction patterns and genetic variations in exon 2 of the *Ovar-DRB1* gene in Makuie sheep were dramatically high.

The results showed that the most of the studied animals carry alleles A, O, Q, and P, and a few carry allele B. Only animals with genotype AB had significant differences (P

≤ 0.05) in weight at 9 months and weight at 12 months as compared to other genotypes. Among 18 genotypes observed, 7, 6, 4, and 5 genotypes contained alleles A, O, Q, and P, respectively. Only 3 genotypes contained allele B (Table 2). As significant difference was found between the AB genotype and AE, AP, AQ, and other genotypes in weight at 9 months and weight at 12 months, we conclude that allele A was not solely effective on these traits; on the contrary, alleles A and B together affected the traits. None of genotypes identified in the present study were involved in birth weight and weight at 3 and 6 months. We expect that sheep carrying the AB genotype will show a significant difference just after weaning in weight gain compared with sheep having other genotypes. The lack of significant effects of this genotype on birth weight, weaning weight, and weight at 6 months is probably due to genetic weakness of sheep at birth.

The effects of genetic variation in the *MHC-DRB1* gene on growth and reproduction traits have been investigated in experimental flocks of 7 Merinoland rams and 249 ewes and their offspring (381 lambs) from consecutive lambings. A total of 16 alleles were detected and a general mixed model revealed that the presence of a 411-bp allele is associated with increasing fertility (lambs born and pregnancy status) and a 349-bp allele is significantly associated with birth weight (9). Association between serologically defined class I polymorphism and rate of growth in cattle has been reported by (30), which points to the more or less indirect effects of MHC on growth traits.

In conclusion, this study was the first attempt to examine any polymorphism at exon 2 of the *Ovar-DRB1* gene in the Iranian Makuie sheep breed. The relationship between polymorphism at exon 2 of the *Ovar-DRB1* gene with weight traits in Iranian Makuie sheep was determined. Further studies with numerous markers and genes in the Ovar-MHC region and other breeds will be required in order to understand MHC genetics in sheep and clarify the genetic background of immunological influences on growth traits.

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