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A Preliminary Study of Marker Data Applicability in Gene Introgression Program for Afshari Sheep Breed

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Abstract: The traits with the greatest financial impact on sheep production are the number of lambs weaned per ewe and growth efficiency. *FecB* and *Callipyge* are the most well known major genes concerned with these traits. In order to conduct a Marker Assisted Introgression (MAI) program we surveyed the presence of responsible mutations in Afshari sheep breeding flock. Direct tests to detect of $FecB^B$ and *CLPG* alleles were conducted on 74 and 58 DNA samples respectively by PCR-RFLP assay. Also the polymorphism content of the breeding flock was investigated by genotyping of one hundred randomly sampled animals at eighteen microsatellite loci selected on the chromosomes carrying and not carrying introgressed genes. A part from MCMA26 monomorphic pattern, microsatellite loci showed moderate level of polymorphism, as such totally 102 alleles were detected with a mean number of 6 alleles per locus. A full characterization of this set of seventeen polymorphic loci was carried out generating allele frequency distributions that were used to estimate genetic parameters of these loci, including expected heterozygosity, Polymorphism Information Content (PIC), probability of identity (I) and discrimination power (D). The average expected heterozygosity was 0.72 (SD = 0.07) and the average PIC was 0.67 (SD = 0.08). Cumulative value of I considering for linked loci were estimated as 3.128E-13. The results of microsatellite analysis indicated that, despite the selective breeding and closed flock system over a number of generations, a relatively high level of heterozygosity still exists in the breeding flock. Besides, the banding patterns resulted from *AvaII* digestion of *FecB* and *CLPG* amplicons approved the absence of the mutations in this flock. These observations demonstrate applicability of marker data and would assist to make decision on starting MAI program for inclusion of *FecB* and *CLPG* genes into the Afshari breed.

Key words: Afshari sheep, *FecB*, *Callipyge*, microsatellite, MAI

INTRODUCTION

The trait with the greatest financial impact on sheep production is the number of lambs weaned per ewe (Bradford, 2002). Correspondingly, breeding schemes for improved lamb-meat production have focused on selecting animals with superior reproductive capacities and studies directed at the genetic improvement of sheep have been primarily concerned with reproductive traits (Montgomery *et al*., 2001). Another trait could be important in sheep breeding is improvement of growth efficiency. Achievement of increased feed conversion efficiency can be attained through the use of hormonal growth promoters, transgenic animals, nutritional strategies, choice of terminal sire breed and marketing lambs at appropriate slaughter weights (Sillence, 2004). However, the banning of hormonal growth promoters by the European Union, the problems with gene expression in transgenic animals and the limited advance of nutritional studies in sheep have restricted the incorporation of these strategies into production schemes (Coeckett *et al*., 2005).

In the early 1982 and soon after in 1983 two mutations named *FecB* and *Callipyge* appeared in two commercial Booroola and Dorset sheep flocks, respectively in New South Wales Australia and Oklahoma State, USA (Piper and Bindon, 1982; Cockett *et al*., 1999). Ewes inheriting one copy of the Booroola mutation from either parent gave birth to about 1.0 extra lamb per ewe lambing. Homozygous carriers produced about 3.0 extra eggs resulting in about 1.5 extra lambs per ewe lambing (Montgomery *et al*., 1993). The low heritability of fertility traits and the desire to produce more lambs per ewe from meat and dairy breeds has lead to many crossbreeding

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programs seeking to obtain the benefits of the Booroola gene (Gootwine *et al.*, 2001, Southey *et al.*, 1995, Pardeshi *et al.*, 2005, Mishra *et al.*, 2006, Farquhar *et al.*, 2006).

Sheep expressing *Callipyge* mutation exhibited marked enlargement or hypertrophy of certain muscles, notably those of the hind legs and loin. The total weight of excised muscles from the pelvic, torso and thoracic limbs was greater in callipyge lambs by 42, 50 and 14%, respectively, than in normally muscled lambs (Jackson *et al.*, 1997b). Interestingly this muscle hypertrophy develops after about a few weeks of age (Jackson *et al.*, 1997a), so there is no increased risk of dystocia for callipyge lambs. In addition, *Callipyge* lambs exhibit superior feed efficiencies and lower daily feed intakes (Jackson *et al.*, 1997a), which result in lower production expenses. Therefore, the widespread production of *Callipyge* lamb would have the potential to lower the cost of lamb for consumers and to increase the profitability of the sheep industry.

The main purpose of sheep industry in Iran is meat production and Afshari sheep is one of the most important meat breeds in the country. Over the past years steps have been taken towards genetic improvement of production traits focusing on meat production over breeding flocks in custody of Agricultural-Jihad ministry. This breed has a relatively good performance in milk traits, but from the prolificacy point of view its rate of twin birth is just about 10%. As lamb production is an important source of income, increasing the fecundity has been an important breeding goal of the Afshari sheep. Agricultural-Jihad Ministry of Iran has provided a grant for a breeding program aimed to improve the prolificacy of Afshari sheep breed through the inclusion of the B gene of the Booroola sheep. Considering some desirable production characteristics of *Callipyge* gene, joint inclusion of these alleles can also help to approach breeding aims of the sheep. As mentioned, up to now some backcrossing programs have been conducted to inclusion of *FecB* gene into a number of sheep breeds throughout the world. However, all of them had been planed based on traditional procedures and limited use of marker data.

Discovery of polymorphic microsatellite DNA markers-segments of the nuclear genome composed of tandem repeat of short-sequence motifs-facilitated using of these markers to construction of genetic maps in domestic animals, including sheep, cattle and other ruminants (Maddox *et al.*, 2001; Jenkings *et al.*, 1997). Many studies have been undertaken with DNA microsatellites in the selection of animals in breeding programs, a process known as marker-assisted selection (Kashi *et al.*, 1990; Lande and Thompson, 1990). Also the use of those DNA microsatellites in process of Marker-Assisted Introgression (MAI) is under increasing consideration for the purpose of livestock improvement. Hillel *et al.* (1990) suggested that a large battery of genetic markers scattered throughout the genome could be used to select BC progeny containing a greater than expected fraction of the recipient genome. This can be denoted background selection, as opposed to the foreground selection considered by Young and Tanksley (1989). Hospital and Charcosset (1992) surveyed the use of polymorphic markers in gene introgression programs. They simulated situations where only one gene of interest from a donor is introgressed into the genome of a selected recipient by recurrent backcrosses to the recipient genotype. Based on the results of this simulation study, we proposed a crossbreeding plan for introgression of *FecB* gene into Afshari sheep. In this plan, in each generation of the breeding program, the offspring that carry the introgressed gene (detected by PCR-RFLP assay) are chosen and then among these, those carrying the lowest proportion of donor genes at other loci (detected by microsatellite data) are selected.

Consequently, we will be able to remove the donor genome using marker data and to develop a new Afshari strain that carries the fecundity gene. To conduct such a program, it is first necessary to gain knowledge on the absence of the gene in this sheep. Secondly, to hasten the recovery of the recipient genome through backcrossing program, we also need a large battery of polymorphic loci all over the genome as well as linked to genes on surrounding chromosomes. The present study as a preliminary work for the main project, aims (1) to search for responsible mutations and (2) to investigate DNA variability and polymorphism content necessary to genome recovery in Afshari sheep breeding flock.

MATERIALS AND METHODS

Blood sample collection and DNA preparation: Peripheral blood samples taken from one hundred pure breed Afshari sheep belonging to the Research Farm of the University of Zanjan, Agricultural Faculty, were used as material. This breeding flock was established in 1997 by collecting animals from distribution area of Afshari sheep in Zanjan province and was kept fully pedigreed until this time. Blood samples stored in -20°C until DNA extraction. DNA was extracted from 1 mL whole blood samples by salting out method according to Miller *et al.* (1998) with minor modifications.

Direct test for *FecB* gene: A total of 74 blood samples from 31 single birth ewes (at least two records) and 43 twin birth ewes (at least one record) were taken. Direct test to detect of B allele were carried out using the PCR based restriction fragment length polymorphism (PCR-RFLP) method by a primer set, 5′-CCA GAG GAC AAT AGC AAA GCA AA-3′ and 5′-CAA GAT GTT TTC ATG CCT CAT CAA CAC GGT C-3′ previously described by Davis *et al.* (2002). Each 15 μL PCR reaction contained 50 ng template DNA, 200 μM each of dNTP, 4 pmol each primer, 1.5 mM $MgCl_2$, 1X PCR standard reaction buffer and 1 U *Biotherm* DNA polymerase. The amplification was carried out using 35 cycles at 94°C for 15 sec, 60°C for 30 sec and 70°C for 30 sec followed by 72°C for 5 min and 99°C for 15 min. The 190-base pair (bp) product was then treated overnight using *Ava*II (5U) and the resulting products were separated by electrophoresis on a 7% polyacrylamid gel and visualized with silver nitrate.

Direct test for *Callipyge* gene: A total 58 blood samples from 14 sires and 44 ewes were taken. Detection of *CLPG* mutation was carried out using the PCR-RFLP method and a primer set, *CLPG* F: 5’-GGA ATC ATC GTG TCC TGG TC-3’ and *CLPG* R: 5’-CCA GCA GGA TAC TCC GTG TC-3’ described by Coeckett (2005) (Personal communication). Polymerase chain reactions were carried out in 15 μL volume containing approximately 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100), 2 mM $MgCl_2$, 200 μM each dNTP, 2.0 μM each primer, 50 ng ovine genomic DNA and 1 U *Biotherm* DNA polymerase. PCR conditions were as follows: 1 cycle 95°, 5 min; 58°, 2 min and 72°, 2 min, 30 cycles 94°, 30 sec; 58°, 30 sec and 72°, 1 min; hold 4°. The PCR products were then digested overnight using *Ava*II (5U) and the resulting products were separated by electrophoresis on a 7% polyacrylamid gel using silver staining.

Microsatellite genotyping: Eighteen microsatellite markers distributed across the ovine genome were selected as a representative sample of about 100 polymorphic loci predicted for the MAI project. A random sample consisting of one hundred animals were selected to analysis the DNA variability of the flock. The primer pairs were selected from Melbourne university sheep genome databases available on the website: http://rubens.its.unimelb.edu.au/~jillm/jill.htm. The microsatellite loci LSCV43, BM2508, BM143, OarHH55, OarAE101, were selected based on proximity to *FecB* gene on chromosome 6 and three markers CSSM18, OY3 and MCMA26 are flanked to *CLPG* gene on chromosome 18. Others 11 markers are mapped on different chromosomes. Some of subjected microsatellites are also belonging to the panel of FAO/ISAG recommended markers. Chromosomal location and some other characteristics of loci studied are described in Table 1. The standard PCR analysis of microsatellite markers was carried out by loading on standard 7% polyacrylamide non denaturing gel using silver staining.

Statistical analysis: Genotypes were assigned for each animal based on allele size data. The most common measures of genetic diversity such as allelic diversity, heterozygosity and proportion of polymorphic loci were considered. The effective allele number (estimates the reciprocal of homozygosity) was calculated according Hartl and Clark (1989). Observed (H_{obs}) and Nei unbiased expected heterozygosity (H_{exp}) were estimated for all loci (Nei, 1978). These parameters were statistically analyzed using PopGene software package version 1.31 (Yeh *et al.*, 1999). Polymorphism Information Content (PIC) (Botstein *et al.*, 1980) values were estimated in order to assess the relevance of each locus for linkage analysis. We also calculated probability of identity as individual differentiation parameter using the following formula:

$$I = \sum_i p_i^4 + \sum_i \sum_{j>i} (2p_i p_j)^2$$

(Paetkau *et al.*, 1995), where p_i and p_j are the frequencies of the ith and jth alleles in the population. If I is the confusion probability, i.e. that two randomly chosen individuals from a sample have identical banding patterns, then discrimination power (D = 1-I) represents the probability that two randomly chosen individuals have different patterns and thus distinguishable from one another. Cumulative values for two later subjected parameters were estimated as the sequential product of parameter values at each microsatellite locus. Also on the basis of allele and genotype frequencies, a likelihood ratio test (G^2_T) was conducted to test for deviations from Hardy-Weinberg equilibrium (Guo and Thompson, 1992).

RESULTS AND DISCUSSION

Search for *FecB* mutation: There was no difference between banding pattern from ewes having single and twin birth records (Fig. 1). As such, a 190 bp amplified fragment without any enzyme digestion was shown for all ewes, resulting the restriction site surrounding an A→G transition at position 746 of BMPR1B gene, substituting the glutamine with an arginine (CAG-CGG, Q249R)

Table 1: Number of detected alleles (n), effective allele number (ne), observed (H_{obs}) and expected (H_{exp}) heterozygosities per locus, PIC value, probability of identity (I) and discrimination power (D) are presented

Locus	n	ne	H_{obs}	H_{exp}	PIC	I	D	Chr. No	Reported allele size (bp)	Observed allele size (bp)	Temperature (°C)
OarHH55	5	2.71	0.63	0.65	0.58	0.18	0.92	6	117-155	111-127	62
OarAE101	6	2.94	0.83	0.76	0.70	0.10	0.90	6	99-123	106-128	63
BM143	5	2.43	0.65	0.68	0.61	0.16	0.94	6	102-128	107-117	61
BMS2508	7	2.38	0.76	0.70	0.65	0.13	0.87	6	158	154-188	58
LSCV43	7	3.22	0.90	0.76	0.71	0.09	0.91	6	110-130	101-119	52
CSSM18	4	2.32	0.40	0.61	0.54	0.21	0.79	18	116-134	112-118	58
OY3	6	2.22	0.66	0.68	0.67	0.11	0.89	18	160	118-185	57
DYMS1	6	5.52	0.74	0.81	0.80	0.05	0.95	20	159-211	147-210	59
OarFCB304	5	2.43	0.71	0.68	0.62	0.16	0.84	19	150-188	118-148	63
OarAE64	4	3.86	0.51	0.62	0.61	0.15	0.85	7	122-158	116-148	55
OarCP26	6	2.56	0.82	0.71	0.65	0.14	0.86	4	120-170	145-201	55
MCMA2	9	5.21	0.72	0.80	0.78	0.05	0.95	13	157-201	160-195	52
MAF64	7	6.11	0.80	0.83	0.81	0.04	0.96	1	109-141	113-147	63
OarJMP58	9	7.12	0.91	0.85	0.84	0.03	0.97	26	133-159	143-178	52
OarJMP29	7	3.58	0.86	0.72	0.68	0.11	0.89	24	96-150	90-141	58
MAF65	5	3.21	0.85	0.69	0.64	0.15	0.85	15	123-135	129-145	60
BM8125	4	3.36	0.82	0.70	0.58	0.19	0.81	17	116-122	112-123	55
Mean	6	3.60	0.74	0.72	0.67	0.11	0.88				
SD	1.54	1.48	0.13	0.07	0.08	0.05	0.05				

(Wilson *et al*., 2001; Mulsant *et al*., 2001; Souza *et al*., 2001) was not present in Afshari sheep experimental population.

Search for *Callipyge* mutation: As shown in Fig. 1 214 bp product was amplified in Afshari sheep genome. There was no difference between digestion patterns and all sampled animals displayed NN genotype. As such, two 130 and 84 bp amplified fragments from enzyme digestion were observed for all animals, indicating that the restriction site surrounding an A → G transition as functional mutation of callipyge phenotype was not present in the studied animals.

Microsatellite analysis: All eighteen markers subjected in this research were amplified in Afshari sheep. Microsatellite marker MCMA26 showed monomorphic pattern. As many population studies in the literature are based on polymorphic data only, it was necessary to exclude monomorphic data from our estimates for comparison with results of these studies (Bowcock *et al*., 1991; Kidd *et al*., 1991; O'Brien *et al*., 1994). Table 1 summarizes the statistics of genetic variation. Disregarding a monomorphic locus, a total of 102 alleles were detected with the mean 6 alleles per locus. Polymorphism was low at CSSM18, OarAE64 and BM8125 loci, each comprising 4 alleles and moderately high at other loci with up to 9 alleles. While there is no published literature on microsatellite variation in closed breeding flocks for comparison, the allelic variation at the studied loci was comparable those in previous reports available on sheep genome database (Maddox *et al*., 2001). On the basis of this comparison, allelic variability of all loci studied was declined dramatically. As shown in Table 1,

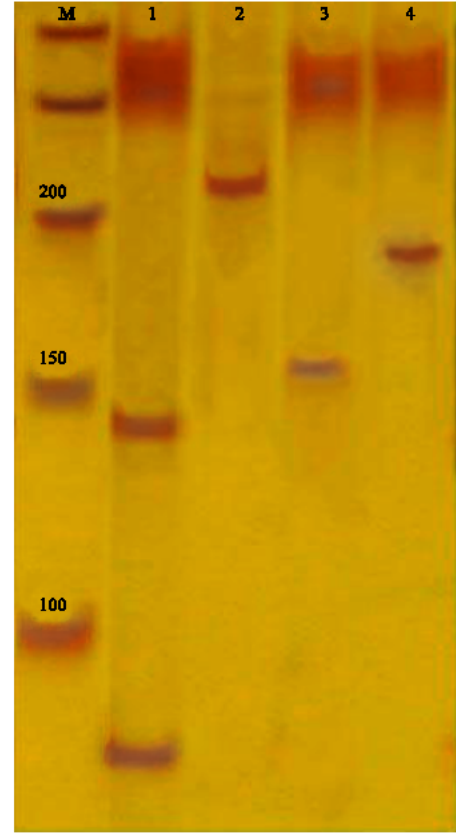

Fig. 1: The banding pattern resulted from *Ava*II digestion of *BMPR1B* and *CLPG* PCR products in Afshari sheep. (M) is 50 bp ladder, (1) and (2) are digested (130, 84 bp) and non digested (214 bp) amplicons of CLPG gene, (3) is 160 bp of digested product of BB homozygote Romney semen and (4) is 190 bp of Afshari sheep +/+ genotype pattern

the allele size range for some loci was not in coincidence with those reported in database, so they can be considered as new alleles for these loci. As a most common parameter for population variability an estimate of both observed (H_{obs}) and expected heterozygosity (H_{exp}) were estimated for each locus (Table 1). Microsatellite marker OarJMP58 having 9 alleles indicated the highest H_{exp} (85%). In contrary, the lowest H_{exp} was shown by CSSM18 locus as 0.61. Mean H_{exp} and mean H_{obs} from data on seventeen polymorphic loci was estimated as 0.72 (SD = 0.07) and 0.74 (SD = 0.13), respectively. The PIC values at each marker were estimated in order to assess the relevance of each locus for linkage analysis. The majority of microsatellite loci would provide an excellent contribution to a genome scan with more than 65% of the meiosis expected to be informative in this population.

We also calculated the individual differentiation parameters such as I and D at each locus (Table 1). These calculations are based on known allele frequency distribution, random mating and linkage equilibrium of studied loci within population. When loci are inherited independent of each other, the total values of individual identification parameters are a sequential product of individual values of the loci. Theoretical cumulative values calculated by this set of markers may not reflect the actual values in a population due to the presence of linkage in some loci (chromosome 6 having five loci and 18 with two loci) and it becomes necessary to also recalculate them. Hence, cumulative values were estimated excluding of linked loci and including only the most effective polymorphic locus at each common chromosome. Consequently the total value for I was estimated as 3.128E-13, hence D was close to 1. The results of likelihood ratio test (G^2_T) for Hardy-Weinberg equilibrium showed significant departures from equilibrium at a 0.05 level of significance for all polymorphic loci. These results were expected, because a number of underlying assumptions of HWE in the breeding flock were violated.

The traits with the greatest financial impact on sheep production are the number of lambs weaned per ewe and growth efficiency. As a preliminary study for a MAI program we searched for *FecB* and *CLPG* responsible mutations in the Afshari sheep breeding flock. The results of PCR-RFLP assay approved the absence of these major genes in the experimental flock. The evidences cleared our doubts on probable presence of the genes in this sheep.

To accelerate the recovery of the recipient genome during backcrosses in MAI program, we have predicted more than one hundred polymorphic loci that will be genotyped throughout the genome, so candidate markers in this study were selected as a representative sample to assess the polymorphism content of the flock which experienced specific selection pressures over a number of generations. All selected microsatellites (ovine, bovine, caprine) were amplified in the Afshari sheep, as such more than 6 alleles were detected per locus in average. With exception of MCMA26 monomorphic locus, seventeen genetic markers have been shown to be reproducible, informative and robust, having the information content necessary for powerful analysis of segregation through MAI program. Assessed results for polymorphism information content, probability of identity and discrimination power are presented in Table 1. It must be noted, the estimated values of D at individual locus could be used as an index for discrimination of the chromosomal segment surrounding marker locus. So it can be considered as a good indicator for the efficiency of a marker locus in recovery process. PIC-values are also relevant to usefulness of a marker locus for linkage analysis. As shown, for the majority of loci studied, PIC-values and Ds were estimated sufficiently high. Furthermore, for some of loci the observed allelic range didn't conform to those reported in previous studies, so they can be considered as specific alleles and therefore could be remarkably useful for discrimination of Afshari genome through the MAI program. As a result of the observed level of DNA variability, detection of the segregation of chromosomal segments from origin of donor and recipient genome could be feasible confidently. Consequently, generalizing these evidences all over the genome make possible implementing marker assisted selection on genomic segments carrying and not carrying donor genes during the backcross generations.

CONCLUSIONS

This study presents an initial step in investigation of variability at the DNA level within a breeding flock of Afshari sheep. The result of this preliminary study indicates that the *FecB* and *CLPG* mutations did not present in the experimental flock. This result would assist in making decision on starting of introgression program for inclusion of *FecB* gene into the Afshari breed. Considering to desirable production characteristics of *Callipyge* gene, joint inclusion of this gene is proposed in this study. Hopefully, inclusion of these genes will lead to increase the meat production of this breed and help to approach breeding aims of the sheep in the country. The significance of this report is that it offers interesting perspectives for the incorporation of molecular genetic techniques to animal breeding in Iran. In addition the results of this study could provide basic molecular data for the research on germplasm characteristics of Afshari sheep.

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