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Survey of genetic diversity growth hormone and growth hormone receptor genes in Iranian indigenous sheep breed (kordian sheep) using a non-radioactive SSCP.

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ABSTRACT

Evaluation of the genetic diversity for two genes in 100 animals of Iranian indigenous sheep breed (Kordian sheep) was done. A non-radioactive method to allow single-strand conformation polymorphism (SSCP) detection was optimized, starting from genomic DNA and PCR amplification of two fragments: exon 4 of the growth hormone gene and exon 6 of the growth hormone receptor gene. In our study, the fragment exon 4 of growth hormone showed three conformational patterns. Exon 6 of growth hormone receptor showed no polymorphism. The absence of diversity does not imply that genes are not polymorphic. It only means that the primers used do not delimitate a polymorphic region. These Data provide evidence that Iranian indigenous sheep breed (Kordian sheep) has genetic Variability, which opens interesting prospects for future selection program and also preservation strategies. Also our data show that PCR-SSCP is an appropriate tool for evaluating genetic variability

Key words: Single Strand Conformation Polymorphism- SSCP, genetic variability, growth hormone, Kordian sheep

INTRODUCTION

Conservation of animal genetic resources is a topic of discussion since the 1950s (Simon, 1984). Indeed, biological, economical, cultural and emotional reasons are some of the arguments that support this initiative. It is essential to avoid the loss of genetic variability since these resources may be valuable for future breeding requirements (Hodges, 1984).

Genetic variability in indigenous breeds is a major concern considering the necessity of preserving what may be a precious and irreplaceable richness, regarding new productive demands. Conservation should be based on a deep knowledge of the genetic resources of the specific breed. It is therefore important to make efforts in order to characterize genetically indigenous breeds.

Progress in molecular biology brought a lot of information about DNA sequences. Using different techniques of DNA analysis in combination with published existent data, it is possible to identify variability within and between populations. Some of the methodologies more frequently used for the identification of point mutations are DGGE (Denaturing Gradient Gel Electrophoresis) (Fischer, Lerman, 1980), TGGE (Temperature Gradient Gel Electrophoresis) (Riesner et al., 1989), Ribonuclease (Myers et al., 1985) and Chemical (Cotton et al., 1988) Cleavage and SSCP (Single

Strand Conformation Polymorphism) (Orita et al., 1989). SSCP is a simple and reliable technique, based on the assumption that changes in the nucleotide sequence of a PCR product affect its single strand conformation. Molecules differing by as little as a single base substitution should have different conformers under non-denaturing conditions and migrate differently Therefore, those differences can be detected as a shift in the electrophoretic mobility (Hayashi, 1991). Early reports used radioactivity, what limited its widespread use. To simplify and make SSCP analysis more efficient, alternative staining methods with ethidium bromide (Yap, McGee, 1992) and with silver (Ainsworth et al., 1991) were described.
The aim of this study is the evaluation of genetic variability of growth hormone gene using a nonradioactive SSCP protocol. This intends to be a first step for a deeper study of "Kordian Sheep" breed in order to establish a breeding program based on marker-assisted selection.

MATERIALAND METHODS

DNA extraction

100 "Kordian " animals from a flock of the "Shirvan" were analysed. Blood samples (10 ml) were obtained by jugular venipuncture, using vacuum tubes treated with 0.25% ethylenediaminetetracetic acid (EDTA). DNA extraction was performed according to Boom et al. (1998). DNA was verified by electrophoresis on 1 (w/v) agarose gel in 1x TBE buffer. Gels were stained with ethidium bromide (1 mg/ml).

DNA amplification by PCR

Twenty-five ml of polymerase chain reaction (PCR) mixture were carried out in 0.5 ml PCR tubes, using a PCR kit (PCR Universal Kit) with the lyophilized components. Each tube contained 1 units of Taq DNA polymerase, 2.5 mM of MgCl2 and 200 mM of each dNTP.and 10 ml of PCR Diluent. To this mixture, 2ml of each primer (25 ng/µl), 4 ml of DNA (25 ng/ml) were added. The primers for the specific amplification of the two fragments were the ones described by Barracosa (1996).
Amplification was carried out in a Eppendorf Mastercycler gradient. Following a hot start (95 ºC for 5 min), 30 cycles were carried out (95 ºC for 30 seconds, 62 ºC for 30 seconds, 72 ºC for 30 seconds), ending with a 5 min final extension at 72 ºC. For growth hormone receptor gene a different annealing temperature (56 ºC) was used. Amplification was verified by electrophoresis on 1% (w/v) agarose gel in 1x TBE buffer using a 250 bp ladder as a molecular weight marker for confirmation of the length of the PCR products. Gels were stained with ethidium bromide (1 mg/ml).

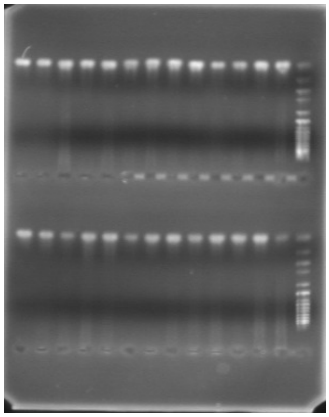
Single-strand conformation polymorphism analysis

PCR products were resolved by SSCP analysis, using denaturing solution 95% of formamide, 1 0 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, plus 20 mM of EDTA, presence 10% of glycerol, voltage (200 to 300 V), running time 4 h and running temperature 4 ºC. Each PCR reaction was diluted in denaturing solution, denatured at 95 ºC for 5 min, chilled on ice and resolved on polyacrylamide gel. The

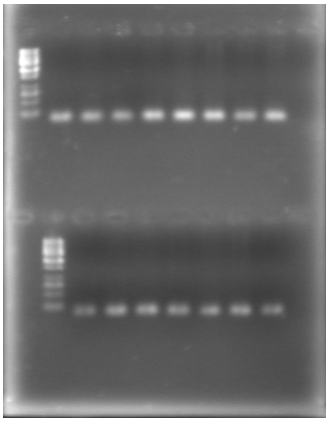
electrophoresis was carried in a vertical unit in 1x TBE buffer. The gels were stained with silver nitrate.

RESULTS AND DISCUSSION

Figure 1 & 2 shows a typical result of PCR amplification of the two analysed fragments.



PCR products of exon 4 analysed by electrophoresis in a 1% agarose gel with ethidium bromide staining



PCR products of exon 6 analysed by electrophoresis in a 1% agarose gel with ethidium bromide staining

Based on the sequence of the GH gene in sheep (Orian et al., 1988), the one set of primer allowed the amplification of a fragment of 214 bp, from nucleotide 1288 to nucleotide 1501, corresponding to exon 4 of the GH gene (Figure 1, primers "5'-CCACCAACC ACC CAT CTG CC-3'" "5'-GAAGGG ACC CAAGAACGC C-3'")(GenebankX12546).

The GH receptor gene has not been entirely sequenced. Based on the work of Adams et al. (1990), we used primers "5'-TTG GCC TCAACTGGACTC TAC T-3'" and "5'-CCACTG GGT CTC ATT TAG TT-3'" for the amplification of a 155 bp fragment (Figure 2) corresponding to exon 6.

After optimization of the parameters that affect the detection of SSCPs, we analysed the PCR products from 100 animals, with the conditions described in table 1

Table 1. Conditions of electrophoresis in SSCP analysis

Gene	Acrylamide	DNA	Denaturing solution	Duration	Time
GH(E 4)	6%	5 µl	10 µl	200	3 h
GH(E-6)	6%	5 µl	10 µl	180	3 h

Figure 3&4 shows the SSCP analysis for the 214 bp fragment of exon 4 of the GH gene. The addition of 10% of glycerol to the gel was favourable in this case, as it allowed a better separation of the single strands. Three conformational patterns were

detected. The frequencies were 75% for pattern 1 and 22% for pattern 2 and 3% for pattern 3.

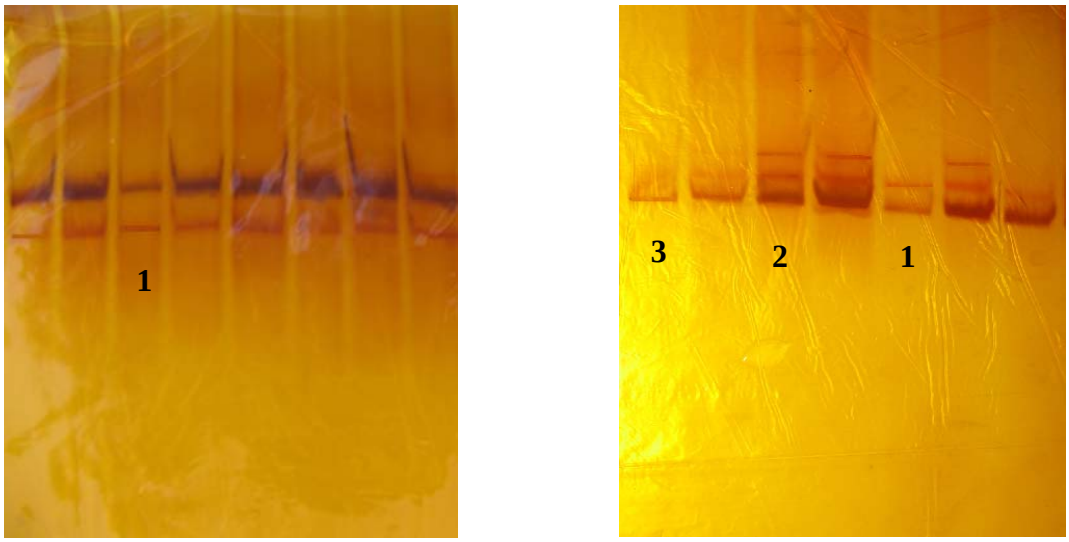


Figure 3&4. SSCP analysis of the 214 bp fragment of GH gene. Electrophoresis was performed in a 6% acrylamide gel with 10% of glycerol, at 200 V and 4 ºC, for 3 h. Three conformational patterns were detected. The frequencies were 75% for pattern 1 and 22% for pattern 2 and 3% for pattern 3.

For the 155 bp fragment of exon 6 of the GH receptor gene, the best results were obtained under the conditions described in table 1 Separation of single strands was clear, and in this case, animals were monomorphic (data not shown).

The maintenance of a great number of local sheep breeds with diversified production conditions offers resistance to the tendency towards the reduction of genetic variability, which affects in larger scale other species (Flamant, 1991). Nevertheless, it is necessary to evaluate the variability within each breed.

Growth in animals is controlled by a complex system, in which the somatotropic axis plays a key role. The genes that operate in the somatotropic axis are responsible for the postnatal growth, mainly GH that acts on the growth of bones and muscles. The growth hormone (GH) gene is candidate for growth in sheep, since that play a key role in growth regulation and development. Effects of GH on growth are observed in several tissues, including bone, muscle and adipose tissue. These effects result from both direct action of GH on the partition of nutrients and cellular multiplication and IGF-1-mediated action stimulating cell proliferation and metabolic processes associated to protein deposition (Boyd and Bauman, 1989).

The effects of growth hormone (GH) polymorphism were investigated in The Canchim beef cattle (5/8 Charolais + 3/8 Zebu). Genotype effects on expected breeding values for birth weight (BW), weaning weight (WW) and yearling weight (YW) were investigated by the least square method. Significant effects

were found for GH genotype on yearling weight (YW) ($p < 0.05$), with positive effects associated with the LV (leucine/valine) genotype (Andréa Pozzi Pereira et al., 2000).

Gootwine et al. (1993) showed that the duplicated gene copy of the ovine growth hormone gene contains a PvuII polymorphism in the second intron. In bovine breeds, GH polymorphisms are extensively studied and some associations between production traits and polymorphism have been made.

Evaluation of the genetic diversity for growth hormone exon 4 in forty animals of the Portuguese indigenous sheep breed (Ovis aries) "Churra da Terra Quente" was done. Growth hormone exon 4 showed two electrophoretic patterns (Estela Bastos et al, 2001).

In our study, exon 6 of GH receptor showed no polymorphism. The absence of diversity does not imply that genes are not polymorphic. It only means that the primers used do not delimitate a polymorphic region.

Our results provide evidence that there is variability within the Iranian indigenous sheep breed "Kordian ". This data opens interesting prospects for future selection programs and also for preservation strategies. Also, our data show that PCR-SSCP is an appropriate tool for evaluating genetic variability. After optimization of the parameters that affect the detection of conformation polymorphisms, this technique is reliable and reproducible. This first molecular approach in "Kordian" intends to be a first step for the genetic characterisation of this indigenous breed. The use of a powerful and reliable molecular technique (such as SSCP) to help breeders in the selection of the animals is the major goal of future research. A larger and more representative sample will allow us to search for possible correlations between productive parameters and genetic variants.

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