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# Rapid PCR-SSCP Screening of Awassi Sheep Using a Short Fragment (260bp) of Leptin Gene

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## ABSTRACT

The genetic variations of Iraqi native sheep breed were described using a non-radioactive single stranded conformation polymorphism (SSCP). Only 260 bp fragment of exon II and a portion of intron II leptin (*LEP*) gene from forty one individuals of Awassi breed were amplified by standard PCR, using the locus specific primers. PCR products were subjected to SSCP and visualized by ethidium bromide. According to SSCP bands, six different genotypes were identified. The resulting genotypes of Iraqi Awassi sheep were AA, AB, AH, AM, AR, and AS, and their alleles were A, B, H, M, R, and S respectively.

**Keywords:** *Awassi, Leptin gene, Sheep, PCR, SSCP Polymorphism*

## INTRODUCTION

One of the symptoms of revolutionizing molecular biology was the development of new sets of genetic markers to genotype animal breeds [1]. Several candidate markers were targeted on the level of DNA architecture, one of these candidate genes was leptin [2]. Leptin word is derived from the Greek term *leptos*, which means "thin" [3]. Leptin was a 16 kD serum circulated protein, it plays a crucial role in the regulation of feed and energy metabolism of the body [4, 5]. However, it was known that leptin was considered as a hormone that regulate the body weight by maintaining the balance between food intake and energy expenditure through signaling to the brain the changes in stored energy levels [6]. In addition to the obvious role of leptin in controlling appetite, it has other roles in regulating growth, reproduction, body composition and immunity, therefore, the *LEP* gene may be a strong candidate gene for evaluation of genetic polymorphism [7]. The size of *LEP* gene was estimated about 20kb and it consisted of three exons separated by two introns, the exons for *LEP* gene cover about 15kb of DNA genome, and the entire coding region of the exon II is separated from the exon III by 1.6kb of intron [8, 9]. Leptin could be considered as one of the best biological markers in mammals since it might play a significant role in "marker assisted selection" [10]. Accordingly, several techniques were employed to exploit the portions of this gene to detect the extent of polymorphisms in a way that could be used in routine labs, such as SSCP [11]. SSCP is a simple [12], sensitive technique [13], and its reliability was confirmed [14]. SSCP is based on the assumption that changes in the nucleotide sequence of a polymerase chain reaction (PCR) product affect its single strand conformation [15]. Molecules differing by as little as a single base substitution should possess different conformers under non-denaturing conditions and migrate differently. Therefore, those differences could be detected as a shift in electrophoretic mobility [16]. Once, the utilization for SSCP polymorphisms could lead to the finding of useful genetic markers of agricultural populations [17], much more attention was gained in *LEP* gene since the control and prediction of obesity is of a high commercial interest [18, 19].
The Iraqi Awassi sheep that belong to one of three native and

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commercial breeds of fat tailed Asian sheep (Karradi, Arrabi, and Awassi) were considered as the most important origins for animal resources in Iraq. However, very little genetic data is currently available about each individual sheep. Thus, employing leptin in the genetic polymorphism in each one of these populations could be considered as an interesting genetic marker. So, as long as the genetic polymorphisms of *LEP* were reported in various domestic livestock [2; 20], the aim of this study was the evaluation of genetic variability of *LEP* in Awassi sheep using a simple and rapid SSCP protocol.

## Materials and Methods

**The experimental animals**

Forty one randomly selected Awassi sheep were chosen in this study. The chosen sheep were bred in the grazing lands that belonging to college of agriculture of Al-Qasim Green University. Standard program of breeding was applied.

**DNA Samples**

Genomic DNA was obtained from the peripheral blood of the jugular vein of Awassi sheep. DNA was isolated using genomic DNA isolation kit (Geneaid Biotech - Taiwan). Purity of DNA was assessed by Nano Drop spectrophotometer, version BioDrop µLITE (Biodrop - UK).

**PCR Amplification**

The *LEP* gene was amplified by PCR using one pair of specific primers. The Primers were selected according to [21] and [22]. The lyophilized primers were purchased from Bioneer (Korea). The sequence of upstream primer was 5'-CGCAAGGTCCAGGATGACACC-3', and the sequence of downstream primer was 5'-GTCTGGGAGGGAGGAGAGTGA-3'. The length of the only amplicon as it was determined by agarose gel electrophoresis was only 260bp, which covered exon II and a portion of intron II of *LEP* gene (data not shown). PCR reaction was performed using *AccuPower* PCR premix (Bioneer - Korea). Each 20µl of PCR premix was contained 1 U of *Top* DNA polymerase, 250 µM of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, 1.5 mM of $MgCl_2$. The reaction mixture was completed with 10 pmol of each primer and 50 ng of genomic DNA. The following program was applied in gradient PCR thermocycler, version; mastercycler-nexus (Eppendorf - Germany); the amplification was began by initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 20 sec, and elongation at 72°C for 26 sec, and was concluded with a final extension at 72°C for 10 min. After performing PCR thermocycling, PCR products were tested by 1.5% agarose gel electrophoresis. It was made sure that all PCR resolved bands are specific and consisted of only one band (260bp fragment each). All faint or non specific PCR bands were eliminated.

**SSCP analysis**

SSCP was performed according to Orita and his colleges [11] with some modifications. Briefly, 10 µl of each amplification product was mixed with equal volume of SSCP denaturing loading buffer (95% formamide, 20 mM EDTA pH 8, 0.05% xylene cyanol and 0.05% bromophenol blue). The samples were heat-denatured at 95°C for 5 min and chilled on ice, and loaded onto 20 x 20 x 0.1 cm gel format (Cleaver Scientific - UK). Denatured PCR products were loaded into the wells of 8% acrylamide/bis (37.5:1), containing 7% glycerol, and 1x TBE buffer. The gel was run under 30 W for about 7 hrs. Gels are stained by ethidium bromide. After the destaining, photos of gels were taken using gel photodocumentation unit, version Chemidoc MP imaging (Bio-Rad - USA).

## Results

According to PCR-SSCP, six conformational patterns of exon II and a portion of intron II in *LEP* gene is resolved in this study as they determined in Awassi samples. However, in present

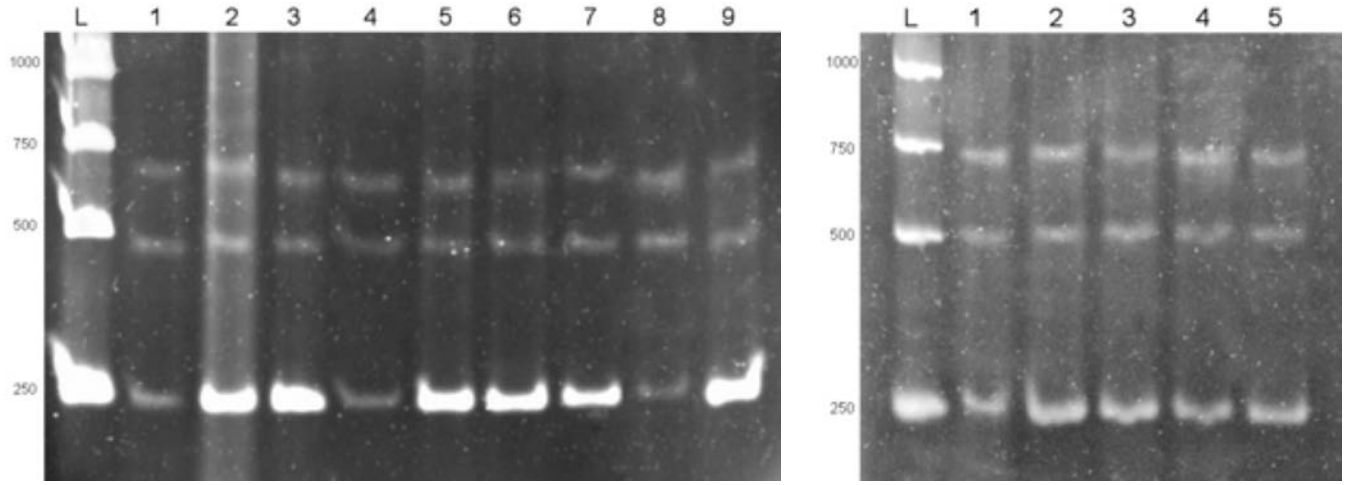


**Figure 1:** AH genotype resolved from SSCP analysis for Awassi samples as it was visualized using ethidium bromide. L; refers to molecular weight ladder. Lanes (1 – 9) in the left gel and lanes (1 – 5) in the right.

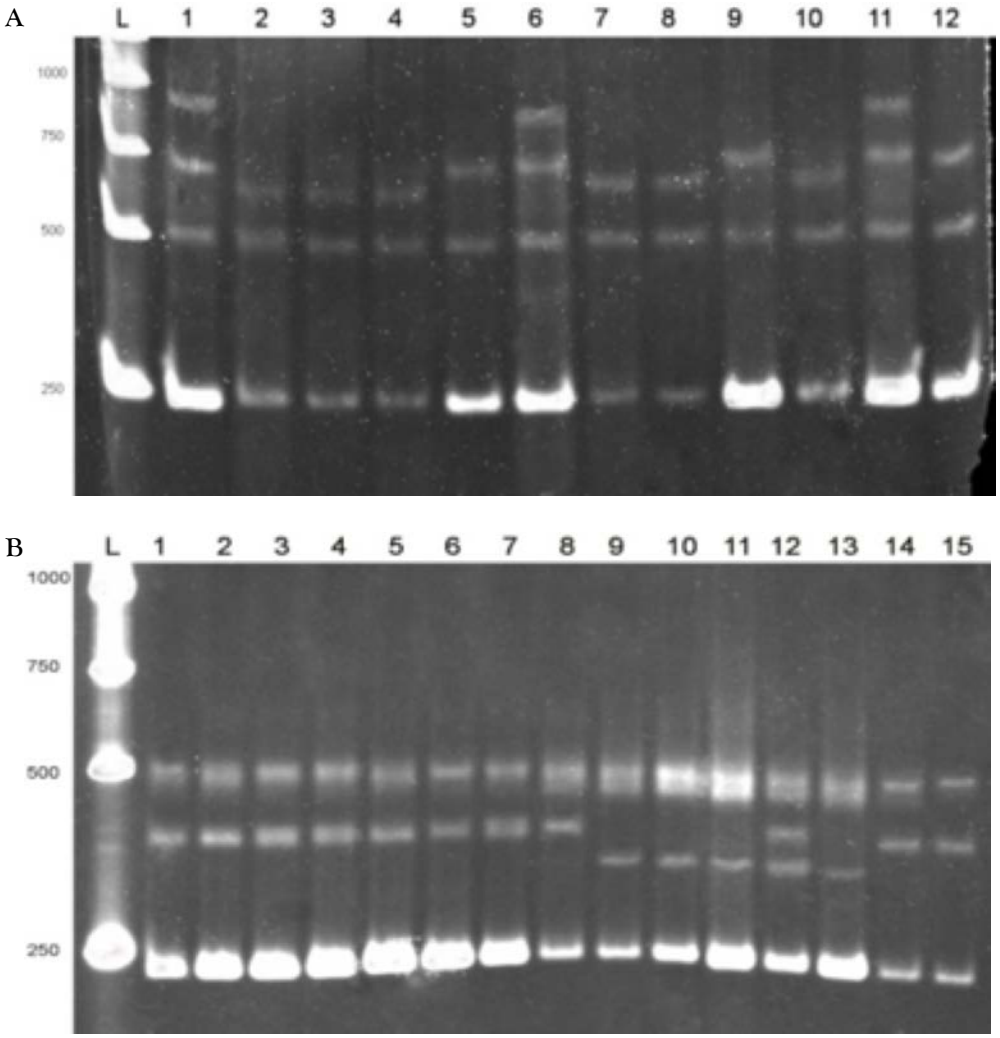


**Figure 2:** six genotypes gel representation according to PCR-SSCP. (a). AH, AA, and AM genotypes resolved from SSCP analysis for Awassi samples as they were visualized using ethidium bromide. L; refers to molecular weight ladder. Lanes (5, 9, and 12) refers to AH genotype, Lanes (1, 6, and 11) refers to AA genotype, and Lanes (2, 3, 4, 7, 8, and 12) refers to AM genotype. (b). AB, AS and AR genotypes resolved from SSCP analysis for Awassi samples as they were visualized using ethidium bromide. L; refers to molecular weight ladder. Lanes (1 – 8, 14 – 15) refers to AB genotype, lane (9 – 11, and 13) refers to AS genotype, and lane (12) refers to AR genotype.

**Table 1: Allele frequencies and diversity measurement of Awassi sheep according to SSCP analysis**

| No | Allelic frequency | | | | | | |
|---|---|---|---|---|---|---|---|
| | A | B | H | M | R | S | Total |
| 41 | 0.5366 | 0.1220 | 0.2073 | 0.0732 | 0.122 | 0.0488 | 1 |
| na | 6 | | | | | | |
| ne | 2.8276 | | | | | | |
| I | 1.3093 | | | | | | |

*na = Observed number of alleles, ne = Effective number of alleles, I = Shannon's Information index*

study, six genotypes (AA, AB, AH, AM, AR, and AS) and six alleles (A, B, H, M, R, and S) were identified for exon II and a part of intron II of *LEP* gene of Awassi sheep. It was revealed that AH genotypes have much more frequencies over other genotypes (Table 1). Moreover, it was noticed that AH genotype consisted of three distinct bands (Fig. 1).

The genotype AH was the predominant one since the number of AH genotype in this study was seventeen samples, which constituted about 41% (Fig. 1 and 2), while the AB genotype was less common since it was included ten samples, which constituted 24%, whereas other samples showed less predominant genotypes (Fig. 2).

Regarding the genetic diversity measures for POPGENE - Version 1.31 [23], the nature of Awassi alleles gave significant differences of the several resolved alleles on the level of 5% (Table 1).

## Conclusion

The variations of LEP gene have been characterized in several domestic animals around the world, but in Iraq, these characterizations haven't been applied in Iraq Awassi sheep till now. These possible data might be attributed to the highly genetic variability for this breed in Iraq. These data were concomitant with the fact of the increasing of genetic diversity as a result of the increasing of number of alleles [24]. However, the determinations of the genetic polymorphism of LEP gene based primers by PCR-SSCP were shown multiple variations in Awassi sheep. The lengths of the obtained bands were found to be partly in accordance to those expected according to the position of the primers designed from the previous studies on other breeds [21, 22].

The currently applied PCR SSCP analysis that was used in this study showed a relatively high level of *LEP* gene polymorphism compared with other *LEP* gene based SSCP studies (Fig. 1 and Fig. 2). These results might be referred to the presence of a wide ratio of hybridization, selection, or genetic diversity for Awassi sheep. Although, no comparable standards were available in this local breed, the results of this study in Iraqi Awassi sheep yielded comparable standards with some sheep breeds in the nearby countries, in which *LEP* gene specific primers were extensively used in genotyping [21, 22].

As it was expected, though the same specific primers were used in all previous cases but the polymorphism obtained was highly dependable on the type of the breed used. Furthermore, the applying of exon III specific primers in other types of sheep were given ten different genotypes for only one breed [20], whereas only five genotypes were resolved in other breeds of sheep [25], and ovine (6). Although the revealed genotypes might be highly variable, the range of the obtained genotypes was between "two" into "ten" different polymorphisms.

Although PCR-SSCP is not the only reliable technique to genotype breeds of different organisms and other available techniques are used instead, such as restriction fragment length polymorphism (RFLP) [26], Denaturing Gradient Gel Electrophoresis (DGGE) [27, 28], and Temperature Gradient Gel Electrophoresis (TGGE) [29], but PCR-SSCP performance is the simplest and cheapest one particularly by direct exposure of PCR products in a high concentrated ethidium bromide solution (30). Moreover, it was reported that this non-radioisotopic technique was quite efficient to play a substitutive role for the cumbersome silver staining or to radioisotopes staining methods in several cases of PCR SSCP [30-32].

According to our knowledge, these PCR-SSCP resolved Iraqi Awassi bands were the first resolved genotypes of Awassi breed in Iraq, since no previous study was used to genetically evaluate this breed in this country. However, no genetic data is currently available to compare different sheep populations in Iraq at least in terms of their SSCP patterns. As long as the utilization of leptin is a suitable and informative genetic marker system to find the evolutionary relationship among closed and inbred population [18], it's possible to apply this promising marker on other breeds as well as Awassi in Iraq. Eventually, in addition to Awassi breed, further investigations including more Iraqi native sheep, such as Karradi and Arrabi breeds will be useful to clarify their origin and any possible genetic relationships among these important three Iraqi breeds using the same *LEP* based PCR-SSCP technique. Nevertheless, further studies are needed to explain the DNA polymorphism and to obtain more precise description about these cases in terms of DNA sequencing studies. It is advisable to apply more markers, in addition to the applied markers, in this breed and some other native breeds such as Karradi and Arrabi in order to uncover their genetic relationships.

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