



RESEARCH ARTICLE

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

ADIL HUSSEIN RADHI¹, ALLAWI LUAIBI DAGHER AL-KHAUZAI², MOHAMMED BAQUR S AL-SHUHAIB^{1*}

¹Department of animal resources/college of agriculture/Al-Qasim Green University, Iraq

²Department of animal resources/college of agriculture/Al-Qadisiyah University, Iraq

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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

*Corresponding Author

Dr. Mohammed Baqur S Al-Shuhaib

Department of Animal Resources, College of Agriculture, Al-Qasim Green University, Iraq.

Tel: +9647707115693

Email: baquralhilly_79@yahoo.com



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 μ LITE (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 20 μ l of PCR premix was contained 1 U of *Top* DNA polymerase, 250 μ M 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 μ l 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

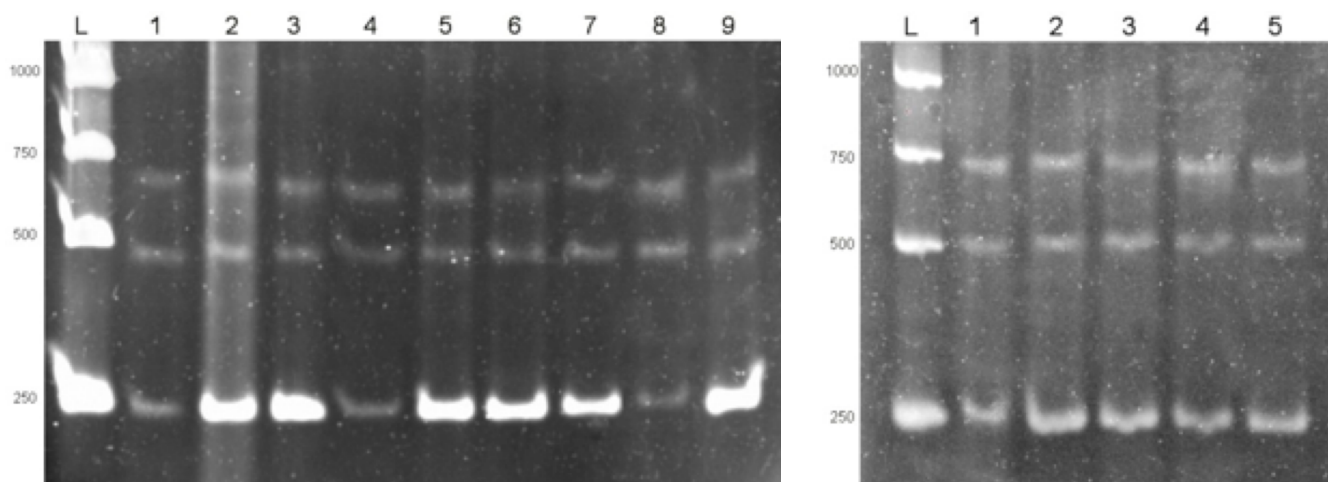


Figure 1: AH genotype resolved from SSCP analysis for Awassi samples as it was visualized using ethidium bromide. L; refers to molecular weight ladder. Lanes (1 – 9) in the left gel and lanes (1 – 5) in the right.

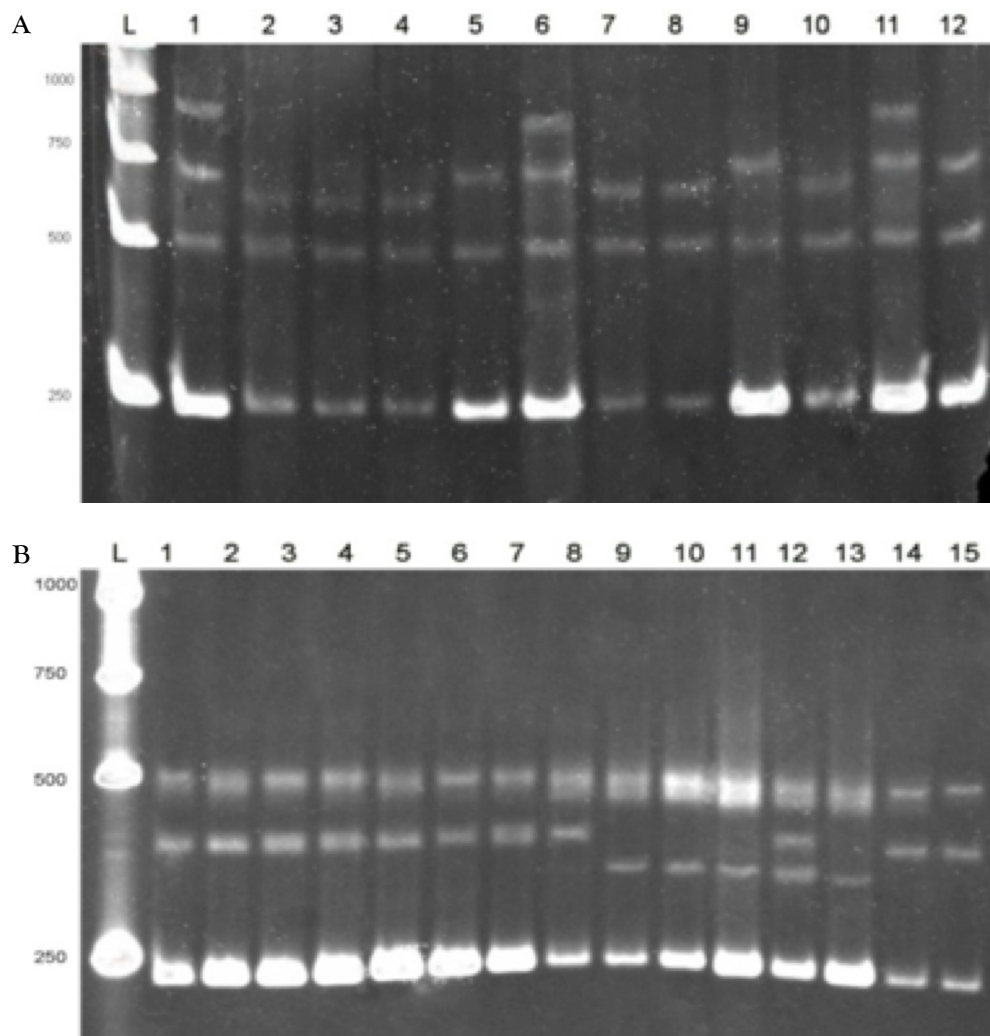


Figure 2: six genotypes gel representation according to PCR-SSCP. (a). AH, AA, and AM genotypes resolved from SSCP analysis for Awassi samples as they were visualized using ethidium bromide. L; refers to molecular weight ladder. Lanes (5, 9, and 12) refers to AH genotype, Lanes (1, 6, and 11) refers to AA genotype, and Lanes (2, 3, 4, 7, 8, and 10) 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						Total
	A	B	H	M	R	S	
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 Iraqi 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.

REFERENCES

1. Kale DS, Yadav BR, Prasad I: **DNA Polymorphisms at Candidate Gene Loci and Their Relation with Milk Production Traits in Murrah Buffalo (*Bubalus bubalis*)**. *Iranian Journal of Applied Animal Sci* 2014, **4**: 39-43.
2. Nassiry MR, Heravi Moussavi A, Alashawkany AR, Ghovati S: **Leptin Gene Polymorphism in Iranian Native Golpayegani and Taleshi Cows**. *Pakist. J. Biol. Sci* 2007, **10**: 3738-3741.
3. Liefers S: **Physiology and genetics of leptin in perparturient dairy cows**. 2004, Wageningen University. *Ph. D. Thesis*.
4. Geary TW, McFadin EL, MacNeil DM, Grings EE, Short RE, Funston RN, Keisler DH: **Leptin as a predictor of carcass composition in beef cattle**. *J. Anim. Sci* 2003, **81**: 1-8.
5. Chilliard Y, Deavaud C, Bonnet M: **Leptin expression in ruminants: nutritional and physiological regulations in relation with energy metabolism**. *Domest. Animal Endocrinol* 2005, **29**: 3-22.
6. Zhou H, Hickford JGH, Gong H: **Identification of allelic polymorphism in the ovine leptin gene**. *Mol. Biotech* 2009, **41**: 22-25.
7. Agarwal R, Rout PK, Singh SK: **Leptin: a biomolecule for enhancing livestock productivity**. *Indian J. Biotechnol* 2009, **8**: 169 - 176.
8. Rock FL, Altmann SW, Heek MV, Kastelein RA, Bazan JF: **The leptin haemopoietic cytokine fold is stabilized by an intrachain disulfide bond**. *Horm Metab Res* 1996, **28**: 649-652.
9. Hiroike T, Higo J, Jingami H, Toh H: **Homology modeling of human leptin/leptin receptor complex**. *Biochem Biophys Res Commun* 2000, **275**: 154-158.
10. Dubey PP, Sharma A, Gour DS, Prashant, Jain A, Mukhopadhyay CS, Singh A, Joshi BK, Kumar D: **Leptin gene polymorphism in Indian Sahiwal cattle by single strand conformation polymorphism (SSCP)**. *South African Journal of Animal Science* 2008, **38** (2): 131-135.

11. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T: Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci. USA* 1989, 86: 2766-2770.
12. Sunnucks P, Wilson AC, Beheregaray LB, Zenger K, French J, Taylor AC: SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. *Mol. Ecol* 2000, 9: 1699-1710.
13. Hayashi K, Yandell DW: How sensitive is PCR-SSCP? *Human Mutation* 1993, 2: 338-346.
14. Jaeckel S, Epplen JT, Kauth M, Mitterski B, Tschentscher F, Epplen C: Polymerase chain reaction – single strand conformation polymorphism or how to detect reliably and efficiently each sequence variation in many samples. *Electrophoresis* 1998, 19: 3055-3061.
15. Girman D: The use of PCR-based single-stranded conformation polymorphism analysis (SSCP-PCR) in conservation genetics. In: *Molecular Genetic Approaches in Conservation* (eds Smith TB, Wayne RK) 1996, 167–182. Oxford University Press, Oxford.
16. Hayashi K: PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods and Applications* 1991, 1: 34-38.
17. Bonifacio C, Santos C, Belo C, Cravador A: Single stranded conformation polymorphism (SSCP) analysis of α S1- casein, β - casein and κ - casein genes in Charnequeira Portuguese indigenous breed. *Arch. Zootec* 2001, 50: 105-111.
18. Aslaminejad AA, Nassiry MR, Farajollahi H, Mahdavi M, Abbasi H, Javadmanesh A: Polymorphism in Exon 3 of Leptin Gene in Iranian Native Cattle Breeds. *J. Appl. Anim. Res* 2010, 37: 159-162.
19. Oikonomou G, Michailidis G, Kougioumtzis A, Avdi M, Banos G: Effect of polymorphisms at the STAT5A and FGF2 gene loci on reproduction, milk yield and lameness of Holstein cows. *Res. Vet. Sci* 2011, 91: 235-239.
20. Shojaei M, Abadi MM, Foze MA, Dayani O, Khezri A, Akhondi M: Association of growth trait and Leptin gene polymorphism in Kermani sheep. *J. of Cell and Mol. Res* 2010, 2, (1): 67-73.
21. Boucher D, Palin MF, Castonguay F, Gariepy C, Pothier F: Detection of polymorphism in the ovine leptin (LEP) Gene: Association of a single nucleotide polymorphism with muscle growth and meat quality traits. *Can. J. Anim. Sci* 2006, 86: 31-35.
22. Barzehkar R, Salehi A, Mahjoubi F: Polymorphisms of the ovine leptin gene and its association with growth and carcass traits in three Iranian sheep breeds. *J. Biotech* 2009, 7 (4): 241- 246.
23. Yeh FC, Yang R, Boyle T: POPGENE: Version 1.31. Microsoft Window-based Freeware for Population Genetic Analysis, University of Alberta. Edmonton, AB, Canada. 1999.
24. Arranz JJ, Bayon Y, San Plimitivo F: Differentiation among Spanish sheep breeds using microsatellites. *Genet. Sel. E.*, 2001, 33 (5): 529-542.
25. Hashemi A, Mardani K, Farhadian M, Ashrafi I, Ranjbari M: Allelic polymorphism of Makoei sheep leptin gene identified by polymerase chain reaction and single strand conformation polymorphism. *Afr. J. Biotechnol* 2011, 10: 17903-17906.
26. Almeida SE, Almeida EA, Moraes JCF, Weimer T: Molecular marker in the LEP gene and reproductive performance of beef cattle. *J. Anim. Breed. Genet* 2003, 120 (2): 106.
27. Fisher S, Lerman L: Separation of random fragments of DNA according to properties of their sequences. *PNAS USA* 1980, 77: 4420-4424.
28. Patino-Garcia A, Sotillo-Pineiro E, Modesto C, Sierrasesumaga L: Screening of the human tumor necrosis factor- α (TNF- α) gene promoter polymorphisms by PCR-DGGE analysis. *Mutation Research/Mutation Research Genomics* 1999, 406: 121-125.
29. Riesner D, Steger G, Zimmat R, Owens R, Wagenhofer M, Hillen W, Vollach S, Henco K: Temperature-gradient gel electrophoresis of nucleic acids: Analysis of conformational transitions, sequence variations, and protein-nucleic acid interactions. *Electrophoresis* 1989, 10: 377-389.
30. Yap EPH, McGee JO'D: Nonisotopic SSCP detection in PCR products by ethidium bromide staining. *Trends Genet* 1992, 8: 49.
31. Hongyo T, Buzard GS, Calvert RJ, Weghorst CM: 'Cold SSCP': a simple, rapid and non-radioactive method for optimized single-strand conformation polymorphism analyses. *Nucleic Acids Research* 1993, 21: 3637-3642.
32. Xie T, Ho SL, Ma OCK: High resolution single strand conformation polymorphism analysis using formamide and ethidium bromide staining. *Mol Pathol* 1997, 50(5): 276-278.

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Authors Column



Mohammed Baqur S. Al-Shuhaib, Ph. D (Biotechnology), University of Babylon, Iraq-2012, M.Sc (Biotechnology) Honors, University of Baghdad, Iraq-2005, B.Sc (Biology) Honors, University of Babylon, Iraq-2001. Specialization; Molecular Biotechnology, Molecular Genetics, Protein electrophoresis, Animal Transgenesis, Recombinant DNA Technology, Academic Staff; College of Agriculture/Al-Qasim Green University. Several international papers and books are published. Strategic plans; establishing of DNA fingerprinting unit and DNA research center/University of Babylon, participating in the establishing of college of Biotechnology/Al-Qasim Green University.



Adil Hussein Radhi, B.Sc (Animal Resources), University of Baghdad, Iraq-1989. Specialization; Animal Breeding, Animal Genetics, Animal Biotechnology. Affiliation; The general company for grain commerce. Several papers are published. Current activity; M.Sc project of genotyping of animal using recent biotechnological facilities.



Allawi Luabi D. Al-Khuzai, Ph.D (Quantitative Genetics), University of Baghdad, Iraq-2002, M.Sc (Animal Breeding), University of Basrah, Iraq-1985, B.Sc (Animal Production, University of Basrah, Iraq-1982. Specialization; Animal Breeding, Quantitative Genetics, Biological Statistics, Animal Nutrition. Academic Staff; College of Agriculture/University of Al-Qadisiya, more than 40 nationally and internationally published papers have been achieved. Many postgraduate programs have been supervised. Strategic plans; establishing of College of Agriculture/University of Al-Qadisiya, participation in the construction of a research station for the breeding and enhancing the traits of sheep, and the development of animal resources field in Iraq.