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SNPs of leptin gene in three Egyptian sheep breeds

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Leptin is expressed predominantly in adipose tissue, has several important physiological functions. DNA samples obtained from three Egyptian sheep breeds namely Barki, Ossimi, and Rahmani. Detected intron 2 of the *Leptin* gene four single nucleotide polymorphisms (SNPs). The sequence analysis between three Egyptian sheep breeds with six alleles of leptin gene revealed that two nucleotide substitutions at nucleotides 99 (A/G) and 249 (C/T). SNP position at nucleotide 99 was detected two alleles (A and G) and subsequently three genotypes are expected (AA, AG and GG). The A allele was more repeated than G allele while the AG genotype was the most repeated in Ossimi, and Rahmani breeds followed by the AA genotype was the most frequent in Barki breed. The other SNP at nucleotide 249 also two alleles were observed (C and T) and therefore three genotypes are expected (CC, CT and TT). Also, C allele was more frequent than T allele. CC genotype was the most frequent in Rahmani breed then the CT in Ossimi breed, however the two genotypes were the similar frequent in Barki breed. There are four possible haplotypes AC and AT in Barki, GT in Ossimi, and GC in Rahmani breeds. DNA sequence from the three breeds were aligned with *Ovis aries* genome revealed that DNA sequences from the three Egyptian breeds were aligned with *Ovis aries* leptin gene sequence on chromosome 4 (OAR4). The results achieved from this study of the *Leptin* gene in three Egyptian sheep breeds showed the existence of polymorphism.

Keywords: SNP, Sheep, Leptin gene, Genotyping

INTRODUCTION

Sheep are important farm animals and contribute to the living of human populations. Increasing the human population forms more needed for these animals and their products. These requirements can efficiently be resolved by increasing the reproductive capacity and productivity of the animal (Kosgey and Okeyo, 2007).

Studied characterization and recognition of genetic differences among sheep breeds will support in the genetic improvement programs. The molecular genetics application for genetic improvement depend on the ability to genotype individuals for specific genetic loci. The information interest from specific genes in breeding programs has prospect to substantially increase the accuracy of selection and increasing selection differences (Missohou et al., 2006).

Leptin (LEP) is a hormone mainly synthesized in the white adipose tissue in addition to other tissues. The premature and inactive form of leptin protein consists of 167 amino acids, but their mature functional polypeptides consist of 146 amino acids (Marwarha and Ghribi, 2012). Leptin gene DNA sequence consists of 3 exons separated by 2 introns. Out of 3 exons and 2 introns, only two exons are translated into protein (Wallace et al., 2014).

Leptin plays an important role in the energy and glucose homeostasis and plasma glucose levels (Fernandez-Formoso et al., 2015). Leptin can be considered as one of the best biological markers reflecting body fatness both in animals

and human being (Nassiry et al. 2007). Leptin is a hormone which produces 16 kDa non-glycosylated proteins belonging to the class-1 helical cytokine family of proteins (Trombley et al., 2012). Leptin gene was first cloned in mouse and in human 1994 (Zhang et al., 1994, Trombley et al., 2012).

The LEP hormone have an important role in most of angiogenesis, physiological functions; lipogenesis, factor in regulation of appetite (Thomas et al., 2001; Zhou et al., 2009) , milk yield (Komisarek et al., 2005), carcass and meat quality (Schenkel et al. 2005), growth (Kulig and Kmieć, 2009) feed intake (Lagonigro et al., 2003) and fertility (Clempson et al., 2011). Leptin also plays a major role in control of body growth, renal function, haematopoiesis.

The Leptin expression is also modulated according to different physiological and growth stages of animal (Fantuzzi and Faggioni, 2000; Matarese et al., 2005; Trombley et al., 2012 and Wallace et al., 2014). Also, Leptin is linked with other living processes such as bone producing cells, production of blood cells, immune system and reproduction (Henson and Castracane, 2003; Olusi et al., 2003; Hafez, 2013).

Polymorphism in *Lep* gene of ovine has been detected and its linked with growth traits has been reported by (Zhou et al., 2009; Barzehkar et al., 2009; Tahmoorespur et al., 2010; Shojaei et al., 2011; and Hajihosseinlo et al., 2012). Also, Singh et al., (2009) and Wang et al., (2011) described caprine, polymorphism in *Lep* gene.

The present study aimed to investigate the genetic polymorphisms in leptin gene (*Lep*) within three sheep breeds which could be applied as molecular markers in marker assisted selection (MAS) based breeding programs to improve the meat production of three sheep breeds.

MATERIALS AND METHODS

This investigation was carried out in the Cell Biology Department, National Research Centre, Giza, Egypt.

Blood collection and DNA extraction

Sheep Populations: All animals in this study were reared in the Agricultural Experiment Station, Faculty of Agriculture, Cairo University. A total of 90 animals, representing three major Egyptian sheep breeds (Barki, Ossimi and Rahmani). A ten ml of blood samples were collected in sterile 15 ml tubes containing a 0.5ml of 0.5 M EDTA solution (PH 8.0). Genomic DNA was extracted from the whole blood samples according to the method described by Miller et al., (1988) with minor modifications. The DNA concentration was determined using Nano Drop1000 thermo scientific spectrophotometer and then diluted to the working final concentration of a 50 ng/µl.

Animals genotyping and Leptin amplicon sequencing

A DNA fragment which is a part of *Lep* gene was amplified using forward (5-CGCAAGGTCCAGGATGACACC-3) and reverse (5- GTCTGGGAGGGAGGAGAGTGA-3) primer Polymerase chain reaction (PCR) were performed in a 25 µl of reaction volume, which included a 0.2 mM dNTPs and 1.25U of *Taq* DNA polymerase. The Master Mix was aliquot into PCR tubes with 100 ng of ovine DNA. The reaction was cycled with the following conditions; initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation at 94°C, annealing at 60°C and extension at 72°C, each step for 1 min and the final extension for 5 min at 72°C. The PCR products were screened by electrophoresis on a 2% agarose gel in a 0.5X of TBE buffer which stained with ethidium bromide and visualized with an UV transilluminator.

Purification and sequencing of PCR products

The PCR products of selected samples were purified using GeneJET Gel Elution Kit (Thermo Scientific, Dreieich, Germany). The purified PCR products were sequenced using an automated sequencing service (Macrogen, Korea).

Sequence analysis

Sequence analysis was performed using the BLAST program from the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for sequence homology and comparison searches in databases (Altschul et al., 1990). Sequence alignments and comparisons to reveal nucleotide or amino acid substitutions were carried out using Clustal Omega version (1.2.4) (https://www.ebi.ac.uk/Tools/msa/clustalo) as described by (Larkin et al., 2007).

Six Frame Translations of amino acid sequences of each sequence from three Egyptian sheep breeds was carried out using the six frame translation proteins site (http://molbiol.ru/eng/scripts/01_13.html) and/or Open Reading Frame Finder (ORF) site (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).
These sequences which are the most relevant frames were aligned with the amino acid sequences of the corresponding sequences by using the Clustal programs.

The phylogenetic relationships among the Leptin nucleotide sequences or of Egyptian sheep breeds with the other species carried out by using unweighted pair group method with arithmetic mean (UPGMA).

In this study, to identify location of leptin gene from Egyptian sheep breeds in the sheep genome, the *Ovis aries* genome assembly version Oar_v3.1, was used, accessible through the NCBI database (National Center for Biotechnology Information. http://www.ncbi.nlm.nih.gov/).

RESULTS

The fragment of Leptin gene (*Lep*) was amplified from Twelve DNA samples for three Egyptian sheep breeds (four each) namely Barki, Ossimi, and Rahmani used in the present study. The PCR product of 260 bp consisting of exon 2 (from 1 to 72 bp) and part of intron 2 (from 73 to 260 bp) of the *lep* gene.

In order to detect genetic polymorphisms of *lep* gene between the three Egyptian sheep breeds, Clustal Omega was used to align each breed of the three Egyptian sheep breeds with these six alleles of *Ovis aries lep* gene. Genomic sequence of Barki breed samples aligned with six allele of *Ovis aries lep* gene revealed that two sequences (1-Barki and 3-Barki) of them shared high similarity (100%) with LEP-1 allele *Lep* gene, (KC526874) and the other two sequences (Barki-2 and Barki-4) shared high similarity(100%) with LEP-103 A allele *Lep* gene, (KF790578) as shown in Figure 1, phylogenetic tree in Figure 2 and Percent Identity Matrix of DNA multiple sequence alignment in Table 1. Genomic sequence of Ossimi breed samples aligned with six alleles of *Lep* gene revealed that all sequences shared highest similarity (100%) with LEP-3 allele *Lep* gene, (KC526876) as shown in Figure 1, phylogenetic tree in Figure 3 and Percent Identity Matrix of DNA multiple sequence alignment in Table 2. Also, the genomic sequence of Rahmani breed samples shared highest similarity (100%) with *Lep* gene, LEP-2 allele (KC526875) as shown in Figure 1, phylogenetic tree in Figure 4 and Percent Identity Matrix of the multiple sequence alignment in Table 3.

Table 1. Percent Identity Matrix of DNA multiple sequence alignment for the Egyptian sheep breed (Barki) with the six alleles of *Ovis aries* leptin (*Lep*) gene

	1_ Barki	2_ Barki	3_ Barki	4_ Barki	LEP-1	LEP-2	LEP -3	LEP-103	LEP-112	LEP-116
1_Barki_leptin	100.00	99.62	100.00	99.62	100.00	99.62	99.23	99.62	99.23	99.23
2_Barki_leptin		100.00	99.62	100.00	99.62	99.23	99.62	100.00	99.62	99.62
3_Barki_leptin			100.00	99.62	100.00	99.62	99.23	99.62	99.23	99.23
4_Barki_leptin				100.00	99.62	99.23	99.62	100.00	99.62	99.62
LEP-1_allele_ *Ovis*, KC526874					100.00	99.62	99.23	99.62	99.23	99.23
LEP-2_allele_ *Ovis*, KC526874						100.00	99.62	99.62	98.85	98.85
LEP-3_allele_ *Ovis*, KC526874							100.00	99.62	99.23	99.23
LEP-103_ A_allele_ *Ovis*, KF790578								100.00	99.62	99.62
LEP-112_ B_allele_ *Ovis*, KF790579									100.00	99.23
LEP-116_ D_allele_ *Ovis*, KF790580										100.00

Table 2. Percent Identity Matrix of DNA multiple sequence alignment for the Egyptian sheep breed (Ossimi) with the six alleles of *Ovis aries* leptin (*Lep*) gene

	5_ Ossimi	6_ Ossimi	7_ Ossimi	8_ Ossimi	LEP-1	LEP -2	LEP -3	LEP-103	LEP-112	LEP-116
5_Ossimi_ leptin	100.00	100.00	100.00	100.00	99.23	99.62	100.00	99.62	99.23	99.23
6_Ossimi_ leptin		100.00	100.00	100.00	99.23	99.62	100.00	99.62	99.23	99.23
7_Ossimi _leptin			100.00	100.00	99.23	99.62	100.00	99.62	99.23	99.23
8_Ossimi_ leptin				100.00	99.23	99.62	100.00	99.62	99.23	99.23
LEP-1_ allele_ *Ovis*,KC526874					100.00	99.62	99.23	99.62	99.23	99.23
LEP-2_allele_ *Ovis*, KC526875						100.00	99.62	99.23	98.85	98.85
LEP-3_allele_ *Ovis*, KC526876							100.00	99.62	99.23	99.23
LEP-103_ A_allele_ *Ovis*, KF790578								100.00	99.62	99.62
LEP-112_ B_allele_ *Ovis*, KF790579									100.00	99.23
LEP-116_D_ allele_ *Ovis*, KF790580										100.00

On the other way, the sequence analysis between three Egyptian sheep breeds with six alleles of *Lep* gene revealed that two nucleotide substitutions at nucleotide 99 (A/G) and at nucleotide 249 (C/T) both in a part of intron 2, this mutation is considered from the transition type. SNP position at nucleotide 99 was detected two alleles (A and G) and subsequently three genotypes are expected (AA, AG and GG). The A allele was more frequent than G allele, and the AG genotype was the most frequent in Ossimi, and Rahmani breeds followed by the AA genotype was the most frequent in Barki breed, however GG genotype was absent in the three breeds the results of the genotype and allele frequencies for SNP are presented in Table 4 .The other SNP at nucleotide 249 also two alleles were found (C and T) and subsequently three genotypes are detected (CC, CT and TT). Also that the C allele was more repeated than T allele, therefore the CC genotype was the most frequent in Rahmani breed followed by the CT in Ossimi breed, however the two genotypes were the similar frequent in Barki breed, the TT genotypes was absent in the three breeds, results of the genotype and allele frequencies for SNP are presented in Table 4. Each SNP from them have two alleles with respect to two alleles at each of two loci there are four possible haplotypes AC, and AT in Barki GT in Ossimi, and GC in Rahmani breeds.

Sequence comparisons between genomic sequence from the three Egyptian sheep breeds and DNA sequences of *Lep* gene published in data including the six alleles of *Lep* gene **(**LEP-1 allele, LEP-2 allele, LEP-3 allele, LEP-103 A allele, LEP-112 B allele and LEP-116 D allele) in addition to leptin gene in other organism that are available in the GenBank database with accession numbers *Bos taurus* haplotype AC leptin (ob) gene, KC660109; *Bubalus bubalis* leptin variant B gene, JQ043171; *Capra hircus* breed *Sirohi* leptin (LEP) gene, GU944974; *Sus scrofa* leptin gene, U66254; *Canis lupus familiaris* leptin (LEP) NM_001003070; *Camelus ferus* leptin (LEP), mRNA,XM_006184736; *Homo sapiens* leptin (LEP), NG_007450. The results of sequences comparison revealed that DNA sequences from the three Egyptian sheep breeds show high similarity with six alleles of *Lep* gene (99 to100%), also give high similarity with *Bos taurus* haplotype AC leptin (95%);

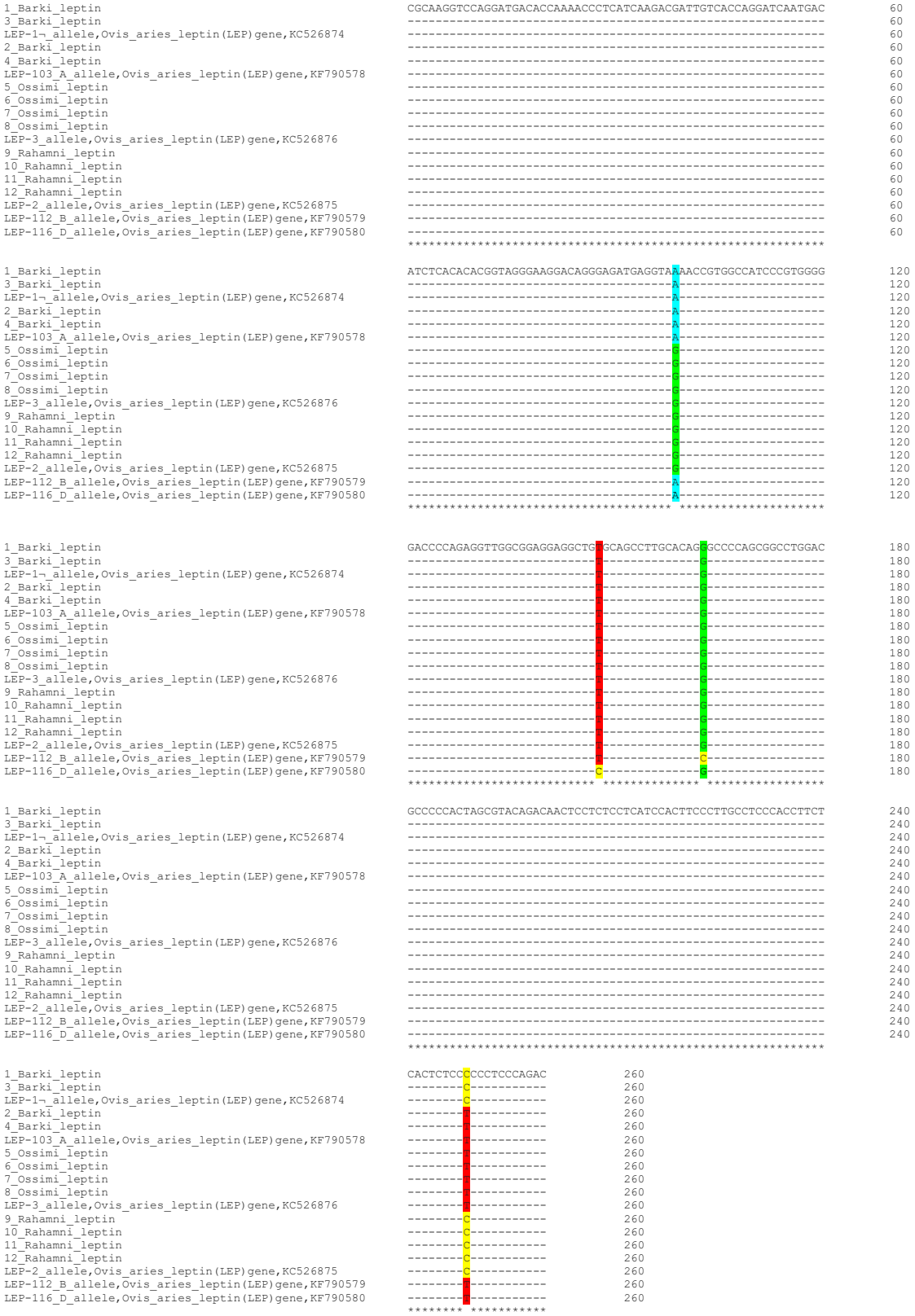

Figure 1: Multiple DNA sequence alignment of the three Egyptian sheep breed (Barki, Ossimi and Rahamni) with the six alleles of *Ovis aries* leptin (*Lep*) gene

Table 3. Percent Identity Matrix of DNA multiple sequence alignment for the Egyptian sheep breed (Rahamni) with the six alleles of *Ovis aries* leptin (*Lep*) gene

	9_Rahamni	10_Rahamni	11_Rahamni	12_Rahamni	LEP-1	LEP-2	LEP-3	LEP-103	LEP-112	LEP-116
9_Rahamni_leptin	100.00	100.00	100.00	100.00	99.62	100.00	99.62	99.62	98.85	99.62
10_Rahamni_leptin		100.00	100.00	100.00	99.62	100.00	99.62	99.23	98.85	98.85
11_Rahamni_leptin			100.00	100.00	99.62	100.00	99.62	99.23	98.85	98.85
12_Rahamni_leptin				100.00	99.62	100.00	99.62	99.23	98.85	98.85
LEP-1_allele_*Ovis*, KC526874					100.00	99.62	99.23	99.62	99.23	99.23
LEP-2_allele_*Ovis*, KC526875						100.00	99.62	99.23	98.85	98.85
LEP-3_allele_*Ovis*, KC526876							100.00	99.62	99.23	99.23
LEP-103_A_allele_*Ovis*, KF790578								100.00	99.62	99.62
LEP-112_B_allele_*Ovis*, KF790579									100.00	98.85
LEP-116_D_allele_*Ovis*, KF790580										100.00

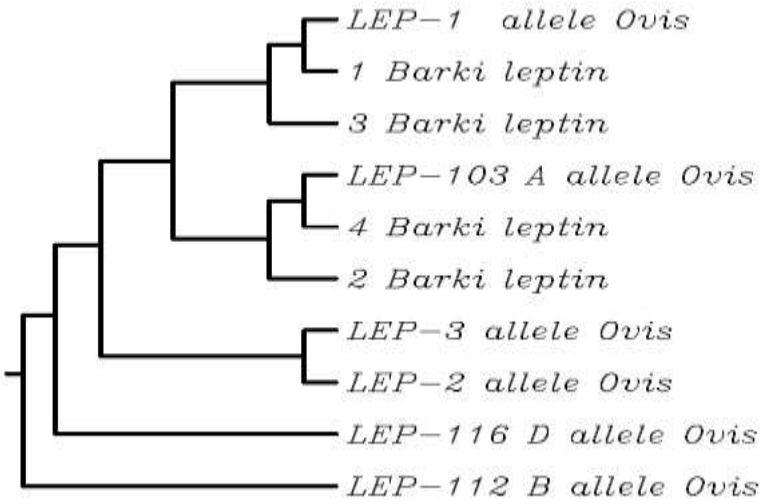

Figure 2: Phylogenetic tree of the Multiple DNA sequence alignment of the Egyptian sheep breed (Barki) with the six alleles of *Ovis aries* leptin (*Lep*) gene.

Table 4. Genotypic and allelic frequencies Leptin gene for the SNPs at the position of nt 99 & 249

Breed	Position	Genotypic Frequency			Allelic Frequency	
Barki	nt 99	AA	AG	GG	A	G
		1.00	00.00	00.00	1.00	00.00
	nt 294	CC	CT	TT	C	T
		00.50	00.50	00.00	00.75	00.25
Osseimi	nt 99	AA	AG	GG	A	G
		00.00	1.00	00.00	00.50	00.50
	nt 294	CC	CT	TT	C	T
		00.00	1.00	00.00	00.50	00.50
Rahmani	nt 99	AA	AG	GG	A	G
		00.00	1.00	00.00	00.50	00.50
	nt 294	CC	CT	TT	C	T
		1.00	00.00	00.00	1.00	00.00

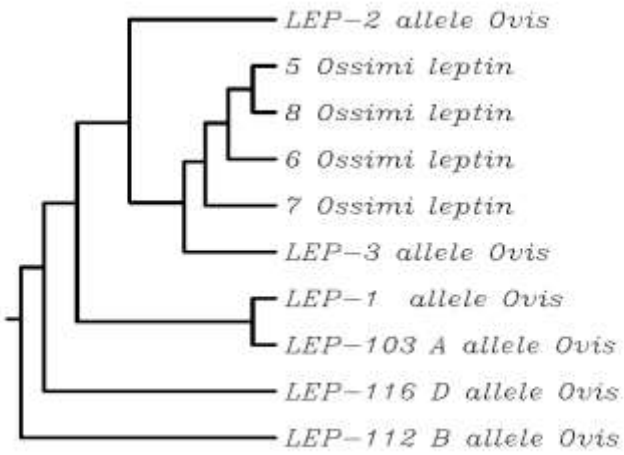

Figure 3: Phylogenetic tree of the Multiple DNA sequence alignment of the Egyptian sheep breed (Ossimi) with the six alleles of *Ovis aries* leptin (*Lep*) gene.

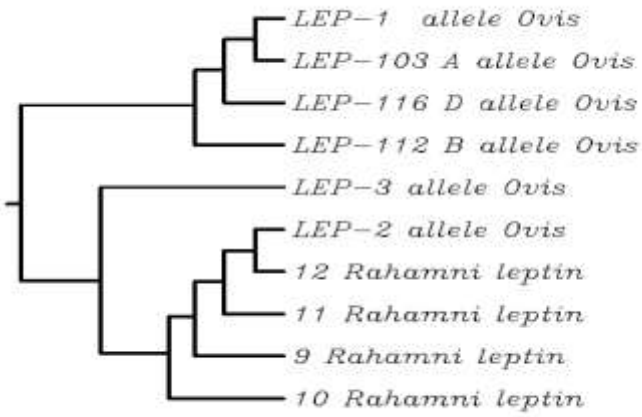

Figure 4: Phylogenetic tree of the Multiple DNA sequence alignment of the Egyptian sheep breed (Rahamni) with the six alleles of *Ovis aries* leptin (*Lep*) gene.

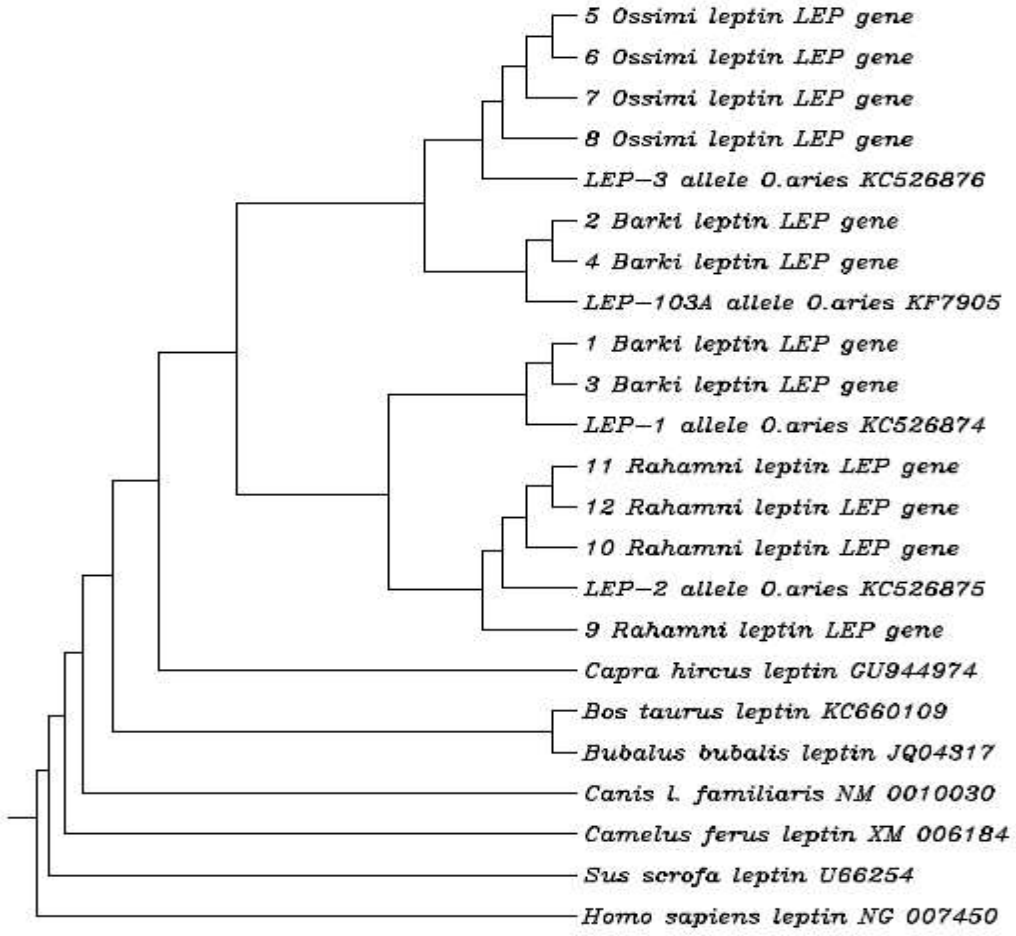

Figure 5: Phylogenetic tree of the Multiple DNA sequence alignment of the three Egyptian sheep breed (Barki, Ossimi and Rahamni) with the six alleles of *Ovis aries* leptin (*Lep*) gene.

Table 5. Percent Identity Matrix of DNA multiple sequence alignment for the Egyptian sheep breed (Barki , Ossimi and Rahamni) with the six alleles of *Ovis aries* leptin (*Lep*) gene and the other sequences of leptin (*Lep*) gene published in GenBank database

	1_Br	3_Br	LEP-1	2_Br	4_Br	LEP-103	5_Os	6_Os	7_Os	8_Os	LEP-3	9_R	10_R	11_R	12_R	LEP-2	*Capra*	*Bos*	Bubalus	*Sus*	*Canis*	*Camelus*	*Homo*
1_Barki	100.00	100.00	100.00	99.62	99.62	99.62	99.23	99.23	99.23	99.23	99.23	99.62	99.62	99.62	99.62	99.62	96.92	94.51	97.70	63.20	57.31	55.00	56.54
3_Barki		100.00	100.00	99.62	99.62	99.62	99.23	99.23	99.23	99.23	99.23	99.62	99.62	99.62	99.62	99.62	96.92	94.51	97.70	63.20	57.31	55.00	56.54
LEP-1			100.00	99.62	99.62	99.62	99.23	99.23	99.23	99.23	99.23	99.62	99.62	99.62	99.62	99.62	96.92	94.51	97.70	63.20	57.31	55.00	56.54
2_Barki				100.00	100.00	100.00	99.62	99.62	99.62	99.62	99.62	99.23	99.23	99.23	99.23	99.23	96.62	94.51	97.70	63.20	56.92	54.62	56.54
4_Barki					100.00	100.00	99.62	99.62	99.62	99.62	99.62	99.23	99.23	99.23	99.23	99.23	96.62	94.51	97.70	63.20	56.92	54.62	56.54
LEP-103						100.00	99.62	99.62	99.62	99.62	99.62	99.23	99.23	99.23	99.23	99.23	96.62	94.51	97.70	63.20	56.92	54.62	56.54
5_Ossimi							100.00	100.00	100.00	100.00	100.00	99.62	99.62	99.62	99.62	99.62	97.31	94.94	97.70	63.60	56.92	54.62	56.16
6_Ossimi								100.00	100.00	100.00	100.00	99.62	99.62	99.62	99.62	99.62	97.31	94.94	97.70	63.60	56.92	54.62	56.16
7_Ossimi									100.00	100.00	100.00	99.62	99.62	99.62	99.62	99.62	97.31	94.94	97.70	63.60	56.92	54.62	56.16
:8_Ossimi										100.00	100.00	99.62	99.62	99.62	99.62	99.62	97.31	94.94	97.70	63.60	56.92	54.62	56.16
LEP-3											100.00	99.62	99.62	99.62	99.62	99.62	97.31	94.94	97.70	63.60	56.92	54.62	56.16
9_Rahamni												100.00	100.00	100.00	100.00	100.00	96.92	94.94	97.70	63.60	56.92	54.62	56.16
10_Rahamni													100.00	100.00	100.00	100.00	96.92	94.94	97.70	63.60	56.92	54.62	56.16
11_Rahamni														100.00	100.00	100.00	96.92	94.94	97.70	63.60	56.92	54.62	56.16
12_Rahamni															100.00	100.00	96.92	94.94	97.70	63.60	56.92	54.62	56.16
LEP-2																100.00	96.92	94.94	97.70	63.60	56.92	54.62	56.16
Capra																	100.00	94.10	96.98	63.03	69.64	74.82	63.27
Bos																		100.00	96.98	69.05	63.50	63.94	63.16
B.bubalis																			100.00	80.68	83.16	84.15	77.61
Sus																				100.00	65.45	77.74	56.99
Canis																					100.00	79.52	68.29
Camelus																						100.00	66.94
Homo																							100.00

1_Barki_leptin (LEP) gene
3_Barki_leptin (LEP) gene
LEP-1_allele, Ovis_aries_leptin (LEP)gene, KC526874
2_Barki_leptin (LEP) gene
4_Barki_leptin (LEP) gene
LEP-103_A_allele, Ovis_aries_leptin (LEP) gene, KF790578
5_Ossimi_leptin (LEP) gene
6_Ossimi_leptin (LEP) gene
7_Ossimi_leptin (LEP) gene
8_Ossimi_leptin (LEP) gene
LEP-3_allele, Ovis_aries_leptin (LEP) gene, KC526876
9_Rahamni_leptin (LEP) gene
10_Rahamni_leptin (LEP) gene
11_Rahamni_leptin (LEP) gene
12_Rahamni_leptin (LEP) gene
LEP-2_allele, *Ovis_aries*_leptin (LEP) gene, KC526875
*Capra_hircus*_leptin (LEP) gene, cds, GU944974
Bos_taurus, leptin, KC660109
Bubalus_bubalis, leptin, JQ043171
*Sus_scrofa*_leptin_gene, complete_cds, U66254
*Canis_lupus*_familiaris_leptin (LEP), mRNA, NM_001003070
Camelus ferus leptin (LEP), transcript variant X1, mRNA, XM_006184736
Homo_sapiens,leptin (LEP) NG_007450

Table 6. Percent Identity Matrix of multiple amino acid sequence alignment for the Egyptian sheep breed (Barki , Ossimi and Rahamni) with the six alleles of *Ovis aries* leptin (*Lep*) gene and the other sequences of leptin (*Lep*) gene published in GenBank database

	Sus	*Homo*	*Camelus*	*Canis*	*Capra*	*Bubalus*	*Bos*	1_Br	2_Br	3_Br	4_Br	5_Os	6_Os	7_Os	8_Os	9_R	10_R	11_R	12_R	LEP-1	LEP-2	LEP-3	LEP-103
Sus	100.00	80.36	91.07	89.29	89.58	87.50	89.58	65.62	65.62	65.62	65.62	65.62	65.62	65.62	65.62	65.62	65.62	65.62	65.62	83.33	83.33	83.33	83.33
Homo		100.00	85.71	82.14	85.71	83.33	83.33	71.88	71.88	71.88	71.88	71.88	71.88	71.88	71.88	71.88	71.88	71.88	71.88	95.83	95.83	95.83	95.83
Camelus			100.00	92.86	95.83	93.75	95.83	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	95.83	95.83	95.83	95.83
Canis				100.00	93.75	91.67	93.75	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	95.83	95.83	95.83	95.83
Capra					100.00	97.92	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Bubalus						100.00	97.92	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Bos							100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1_Barki								100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2_Barki									100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
3_Barki										100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
4_Barki											100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
5_Ossimi												100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
6_Ossimi													100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
7_Ossimi														100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
8_Ossimi															100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
9_Rahamn																100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
10_Rahamn																	100.00	100.00	100.00	100.00	100.00	100.00	100.00
11_Rahamn																		100.00	100.00	100.00	100.00	100.00	100.00
12_Rahamn																			100.00	100.00	100.00	100.00	100.00
LEP-1																				100.00	100.00	100.00	100.00
LEP-2																					100.00	100.00	100.00
LEP-3																						100.00	100.00
LEP-103																							100.00

*Sus_scrofa*_leptin_gene, complete_cds, U66254
Homo_sapiens, leptin (LEP) NG_007450
Camelus ferus leptin_LEP, XM_006184736.2
*Canis_lupus*_familiaris_leptin (LEP), mRNA, NM_001003070
*Capra_hircus*_leptin (LEP) gene, cds, GU944974
Bubalus_bubali, leptin, JQ043171
Bos_taurus, leptin, KC660109
1_Barki_leptin (LEP) gene
2_Barki_leptin (LEP) gene
3_Barki_leptin (LEP) gene
4_Barki_leptin (LEP) gene
5_Ossimi_leptin (LEP) gene
6_Ossimi_leptin (LEP) gene
7_Ossimi_leptin (LEP) gene
8_Ossimi_leptin (LEP) gene
9_Rahamni_leptin (LEP) gene
10_Rahamni_leptin (LEP) gene
11_Rahamni_leptin (LEP) gene
12_Rahamni_leptin (LEP) gene
LEP-1_allele, *Ovis_aries*_leptin (LEP) gene, KC526874
LEP-2_allele, *Ovis_aries*_leptin (LEP) gene, KC526875
LEP-3_allele, *Ovis_aries*_leptin (LEP) gene, KC526876
LEP-103_A_allele, *Ovis_aries*_leptin (LEP) gene, KF790578

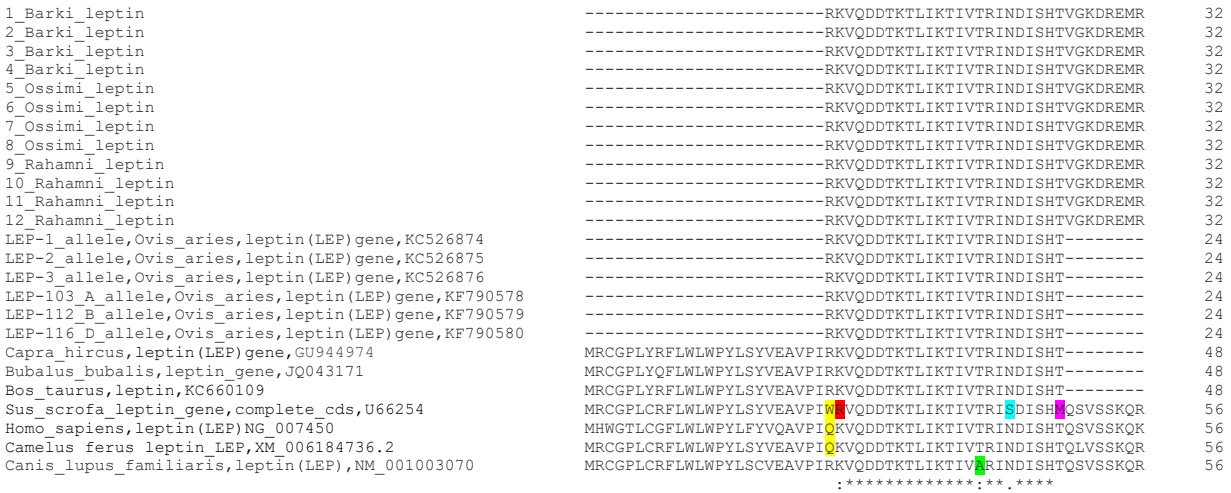

Figure 6: Amino acid multiple sequence alignment of the Egyptian sheep breed with the six alleles of *Ovis aries* leptin (*Lep*) gene and the other sequences of leptin (*Lep*) gene published in GenBank database.

Capra hircus breed *Sirohi* leptin gene and *Bubalus bubalis* leptin variant B gene (97%); but show less similarity with *Sus scrofa* leptin gene (63%); *Canis lupus* familiaris leptin gene (57%); *Camelus ferus* leptin (55%) and 56% with *Homo sapiens* leptin as shown in Table 5 and phylogenetic tree in Figure 5.

Six frame translations of amino acid sequences of each genomic sequence from the three Egyptian sheep breeds was carried out. Translation of 12 Egyptian sheep breeds at this region reported presence of 32 amino acids. These sequences which are the most aligned frames were linked with the amino acid sequences of the relative gene published using CLUSTALW program. For the amino acid alignment, the results show that three Egyptian sheep breed highly similar (100%) to amino acid sequences of the six allele of *Lep* gene, also with *Capra hircus* leptin gene; *Bos taurus* leptin; and *Bubalus bubalis* leptin gene. But less similarity with Sus *scrofa* leptin gene (65%) and 75% for amino acid sequences of *Canis lupus familiaris* leptin gene; *Camelus ferus* leptin and 72% identity with *Homo sapiens* leptin as shown in Figure 6 and Table 6.

DNA sequence from the three Egyptian sheep breeds aligned with *Ovis aries* genome to assign these sequences by using the BLAST program. The results of these alignments represent *Ovis aries Lep* gene sequence on chromosome 4 (OAR4), *Ovis aries* strain OAR_USU_Benz2616 breed Rambouillet chromosome 4, Oar_rambouillet_v1.0 ranging from 101297603 to 101297862. Also, the obtained sequences were submitted to Genbank and have been assigned with accession number MN240008 for *Ovis aries* breed Barki haplotype AC leptin (*Lep*) gene, exon 2 and partial cds; MN240009 for *Ovis aries* breed Barki haplotype AT leptin (*Lep*) gene, exon 2 and partial cds ; MN240010 for *Ovis aries* breed Ossimi haplotype GT leptin (*Lep*) gene, exon 2 and partial cds and MN240011 for *Ovis aries* breed Rahamni haplotype GC leptin (*Lep*) gene , exon 2 and partial cds.

DISCUSSION

Molecular genetics is a significant tool for characterization of genes and identification of genetic variations of individuals (Dekkers, 2002; Naqvi, 2007). Studies on Leptin gene polymorphism and production traits in dairy cattle, sheep and poultry has been reported with promising results and can be considered as one of the best biological markers in animals and human beings (Nassiry et al., 2008).

In this study, the results revealed that Barki breed shared high similarity with two allele Ovis aries *Lep* gene (LEP-1 and LEP-103), Ossimi and Rahmani breed samples showed high similarities with LEP-3, LEP-2 allele *Ovis aries Lep* gene, respectively. The results suggesting that Barki breed represent two alleles however both of Ossimi and Rahmani breed represent only one allele of the *Ovis aries Lep* gene. In sheep, to estimate temporal changes in genetic diversity studied of allelic variation of leptin loci could be used (Nassiry et al., 2008).

On the other way, the sequence analysis revealed that two nucleotide substitutions at nucleotide 99 (A/G) and at nucleotide 249 (C/T), SNPs positions at nucleotides 99 and 249 were detected two alleles at each loci (A and G) and (C and T) subsequently three genotypes are expected (AA, AG and GG) and (CC, CT and TT), respectively. It is reported that the more frequent alleles were A and C than G and T alleles. the most frequent in Ossimi was AG genotype, and Rahmani breeds followed by the most frequent genotype in Barki breed was AA, also the CC genotype was the most frequent in Rahmani breed followed by the CT in Ossimi breed, however the two genotypes were the similar frequent in Barki breed, however GG and TT genotypes were absent in the three breeds. Therefore, two alleles at each of two loci there are four possible haplotypes AC, AT, GT and GC, present in Barki Rahmani and Ossimi breeds. In Barki breed AC haplotype (0.75) found more frequency than AT (0.25) but, they have the same frequencies in Rahmani (0.50) breed the other two haplotypes GT and GC not detected in both breeds. In Ossimi breed the four haplotypes found in the same frequency (0.25).

From the study of the LEP gene in three Egyptian sheep breeds, the existence of polymorphism was detected

Results of this research are most like to published data on other sheep breeds. As we observed two SNPs in intron 2 in these three Egyptian sheep breeds. Also, previous studies in three Iranian sheep populations revealed two SNP in intron 2, according to PCR-SSCP and sequencing of the *Lep* gene at 260 bp PCR amplified product there are exon 2 and part of intron 2 two alleles (A and G) and two genotypes (AA and AG) were observed. Two SNPs in intron 2 were reported by A→G transition at the 113 bp position and T→C transition at the 165 bp position of the ovine *lep* gene (Barzehkar et al., 2009). In other study, single nucleotide polymorphism (SNP) analysis of leptin gene investigated the association of polymorphism with meat quality and skeletal muscle growth .There are three SNPs were detected in the *ovine* LEP gene consisting of two in intron 2 and one SNP in the 3' UTR (Boucher et al., 2006), also intron 2 of the LEP gene, Boucher et al., 2006, identified three SNPs, C→G, C→T and A→G in the Dorset and Suffolk breeds and the genotypic frequencies of AA and AG have been found to be 0.75, 0.25 and 0.87, 0.13, respectively. Two genotypes have been detected (AA and AG) with frequencies ranging from 0.53 to 0.70 for AA and 0.30 to 0.47 for AG genotype in Shal, Zandi, and Zel lambs.

It has been reported in the ovine *Lep* that the total dissected fat gene has no significant association with A→G SNP Boucher et al. 2006. Although in the Zandi breed a significant reduction (1255.3 g) in weaning weight was found ($P< 0.01$) , but no significant association was observed between A→G SNP and carcass traits. Tokuhiro et al. 2003 has been detected that intronic mutations, such as the A→G variant, may affect gene transcription levels and regulation, also effected in splicing abnormalities, which often change the structure of mature protein (Faustino and Cooper, 2003).

In livestock, the *Lep* gene variation has been identified in cattle, pig and Sheep. leptin is informative and a suitable marker system to detect the evolutionary relationships between close populations (Hashemi et al. 2011). In our results, *Lep* gene from three Egyptian sheep breeds have significant homology with sequences of six allele of *Ovis aries Lep* gene, *Capra hircus* breed *Sirohi* leptin gene, *Bos taurus* haplotype AC leptin ~~taurus haplotype AC leptin~~, *Bubalus bubalis* leptin variant B gene, *Sus scrofa* leptin gene, *Canis lupus familiaris* leptin gene, *Camelus ferus* leptin and *Homo sapiens* leptin as 99 to 100%, 97%, 95%, 97%, 63%, 57%, 55% and 56% respectively. Higgins and Sharp 1989 By using CLUSTAL4 algorithm the similarity analysis of the four orthologous LEP genes showed that *Capra hircus* and *Ovis aries Lep* genes are more similar than *Bos taurus* and *Bubalus bubalis* LEP genes (Di Gregorio et al. 2014). Also, pervious study refers to the homology of Leptin gene between different species as buffalo leptin sequence have significant homology with *Ovis aries*, *Bos taurus*, *Capra hircus*, *Bos indicus* and *Homo sapiens* as 99%, 97%, 98%, 97% and 80% respectively. (Datta et al. 2012). Therefore, genetic characterization of leptin gene from Egyptian sheep breeds can also provide valuable information for other species.

The results in this study reported presence of 32 amino acids at this region. The amino acid alignment results showed that three Egyptian sheep breed highly similar (100%) to amino acid sequences of the six allele of *Ovis aries lep* gene, also with *Capra hircus*; *Bos taurus* and *Bubalus bubalis* lep gene. But less similarity with leptin gene amino acid sequences of *Sus scrofa*, *Canis lupus familiaris*; *Camelus ferus* and *Homo sapiens* (65%, 75%, 75% and 72%) identity. The highly similarity between amino acid sequences suggest

that SNPs detected between genomic sequences were non-synonymous. Also, pervious study reported similar result as the amino acid sequence was predict by used the coding regions of the leptin gene.

The phylogenetic tree structure of the complete nucleotide sequence and 504 bp coding region of amino acid sequences of the leptin gene between the mithun gene and 11 other mammalian species, indicated very little difference except for rabbit, mouse, horse and human also showing large differences in the non-coding region of the gene (Dubey et al. 2012).

The length variation of the *Lep* gene within and among species might be due to evolution and differentiation. Many length variations result from insertions and deletions causing amino acid variation within species have been detected by comparison with known sequences (Faith and Owoeye, 2017). The presence of different alleles at a particular Leptin locus is proof of the long-term evolutionary persistence of the locus. This is suggested by the fact that the alleles in one species are often more closely related to the alleles in closely related species than to the other alleles in the same species. The species wise clustering might be due to species specific residues (Takahashi and Nei, 2000) and such patterns of the sequences may be explained by gene conversion and balancing selection.

According to the phylogenetic tree of DNA, and aa sequences of Leptin gene in the different organisms, the locus of *Ovis aries* was related with Lept gene of *Capra hircus*, *Bos taurus*, *Bubalus bubalis* more than of *Sus scrofa*, *Canis lupus familiaris*, *Camelus ferus* and *Homo sapiens*. This clustering based on both of nucleotide and a.a sequences of Lep gene clearly showed that the phylogenetic inter-relationship among these species and is generally in agreement with the known species relationships. In other studies, a phylogenetic tree of the *lept* gene family is depicted. This tree was calculated using MrBayes (Huelsenbeck and Ronquist, 2001) using a previously calculated gene family with multiple sequence alignment (Siltberg and Liberles, 2002). UPGMA tree from the consensus sequence of *Lept* gene showed that the sequence of *Lept* gene of cattle clustered more closely with those of goats than mouse. Sequence of sheep from this figure appeared closer to those of camel than those of swine. Whereas *Lept* gene sequence of horse and rabbit clustered closely than those of swine. In ruminant species, cattle and goats *Lept* sequences clustered closely than those of sheep. While of those of non-ruminant, Leptin sequences of horse and rabbit clustered closely followed by those of swine and then mouse respectively (Akumbugu and Zanwa, 2017). The molecular phylogenetic tree showed the small differences in human, mouse, rabbit and horse, might be result from the alternative evolutionary rate of this gene during evolution (Dubey et al., 2012). So, in our results marked that for studying species classification and phylogenetic relations the leptin gene was appropriate.

The results of DNA sequence alignments of the three Egyptian sheep breeds with *Ovis aries* genome represent *Ovis aries lep* gene on chromosome 4 (OAR4). This result similar to the pervious results as the *Lep* gene maps to chromosome 4 (OAR4) in sheep and to chromosome 4(CHI4) in goat (Perucatti et al., 2006). The Leptin gene is found on chromosome 7q31.3 in humans and on chromosome 8q32 in water buffalo so it highly conserved across species (Vallinoto et al., 2004).

According to analysis of allelic variation of leptin loci, the temporal changes genetic diversity of sheep could be evaluating also that breeds can be differentiated using leptin variability.

CONCLUSION

The SNPs in Leptin gene arise two nucleotide substitutions which detected three genotypes (AA, AG and GG) in two alleles (A and G) for nucleotide substitution at 99, also, the second substitution at 249 arises two alleles (C and T) with three genotypes are expected (CC, CT and TT) in the three Egyptian sheep breeds Barki, Ossimi, and Rahmani.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

KFM designed the experiments and edit the manuscript. HIS and NMS performed the experiments, data analysis and wrote the manuscript.

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