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PCR-SSCP and Sequencing Analysis For Studying Leptin Gene Polymorphism and Its Association with Reproductive Status of Egyptian Buffalo

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LEPTIN gene is one of the potential genes involves intricately in metabolism and growth of animals. Leptin has myriad effect on tissues and endocrine system that ultimately lead to the whole body energy metabolism and has major impact on performance and wellbeing of livestock species. Owing to have key biological features, leptin is one of themost significant candidate gene markers for Marker assisted selection (MAS) studies. This study was performed to identify leptin gene polymorphism and its relation to reproductive status in Egyptian buffaloes. Total of 150 buffaloes were subjected to ultrasound examination for evaluation of reproductive status. Blood samples were collected from all animals and were stored at -20°C till DNA isolation. Amplification of specific fragment 422bp (intron 2) of leptin gene was performed by polymorphism chain reaction (PCR). Single strand confirmation polymorphism (SSCP) was performed on PCR amplicons. Purified PCR amplicons of different electrophoresis patterns were sequenced in both directions using two primers. Sequences results were analyzed using NCBI BLASTn and BioEdit Sequence Alignment software.Two variants(AA and BB) of leptin gene were detected.Genotype AA represented by 64% in fertile buffalo, while its distribution among infertile one was 36%.Genotype BB, was equally distributed by 50% in both fertile and infertile animals. Sequence analysis of both normal and polymorphic one showed different singlenucleotide polymorphisms (SNPs) in leptin gene, but statistically these SNPs had no correlation with reproductive status (fertile or infertile) of examined buffalo.

Keywords: Leptin gene, Buffalo, PCR-SSCP, Sequencing, Polymorphism, Reproduction.

Introduction

Water buffaloes are distributed world wide and mostly found in the South and South East Asia of the world. They are usually grown under extreme conditions. They can benefit from lowquality roughage in swamp and reed areas [1]. Egyptian buffalo populations (water buffaloes) belongs to the Mediterranean buffaloes type. Molecular genetics is a significant tool for characterization of genes and identification of genetic variations of individuals. There has been great interest and urgent need to use the molecular markers in livestock breeding programs. Therefore, using of molecular geneticin formation in breeding programs have made great assistance not only to improve efficiency and adaptability to environmental conditions but also to proceed genetic diversity of livestock animals [2].

Marker assisted selection (MAS) have been widely used to identify the relevance between thegenes and economically important traits of livestock animals. It's wellknown that phenotypic

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characters do not generally represent the real genotypic values of animals. Therefore, using MAS approaches in animal breeding can increase the accuracy of selection. From this opinion, validation of candidate gene markers for MAS is very important to augment genetic gains in breeding. Owing to it has key biological features, leptin is one of the most considerable candidate gene markers for MAS studies [3,4].

Cattle breeds are the most prominent ruminants used in animal genetics studies due to their economic importancein agricultural economy. However, buffalo and cattle have strong genotypic and phenotypic similarities. They are belonging to subfamily of Bovinae [5]. Surprisingly, there were a few genetic studies about characterization of genetic markers that control economic traits in buffalo breeds.

Biological effects of leptin in livestock is similarto human, it plays themain role in regulation and control of productive performanceof animals. Leptin, plays a pivotal role in the control of body growth [6], adaptability, immune function[7], angiogenesis, renal function, hematopoiesis and reproduction. Leptinplays a key role in the regulation of reproductive performance by stimulating GnRH, FSH and LH release, as well asitacts as an autocrine/paracrine stimulator with intissues where it is found in different species[8]. In ruminants, it is clear that leptin gene is expressed in various tissues including adipose, fetal and mammary gland tissues, rumen, and pituitary gland [9]. Thisgene expression is also variable according to various physiological and growth stages of animals. Consequently, leptin could beconsideredas a marker for animal's productivity and health.In dairy animals,the 1st lactation period accompanied with sustainable high milk productionwith a sever mobilization of adipose tissue, resulted in intensedecrease in leptinhormone level which reaches its lower concentration ten days after calving [10]. Liefers et al. [11] reported that as the time be longer to restoreleptin secretion in animal, the longer will bethe duration of the postpartum anestrus. Leptin genewas identified through studies on obese and sterile mice[12] and has been associated with quantitative traits such as carcass fat metabolism [13], energyhomeostasis, milk yield, live body weight and fertility [14].

The leptin gene (obesity gene) was first cloned and discovered in 1994 [12]. Cattle leptin gene has three exonsand two introns (span about 18.9 kb), of which the first exon is not transcribed into protein and located on chromosome 4 [15]. Like cattle, Buffalo leptin gene had similar structure, and the entire coding region is contained in exons 2 and 3, which are separated by a 2 kb intron. (Gen Bank: AH013754.2) and mapped on chromo some 8 (BBU8q32) and consists of 5872bp [16]. Leptin gene shows 67% sequence homology between different species including cow, rat, human, rhesus monkey, orangutan, pig, mouse,dog and chimpanzee species [17]. Since the bovine leptin genehas been recognized, various SNP shave been previously discovered in both introns andexons of the gene among different breeds of cattle.The physiological role and biology of leptin is well reviewed [18,19]. Polymorphic studies on bovine leptin genehave been reported [20-24]. Leptin gene has been mapped to chromosome 7 and 4 inhuman [25] and in bovine respectively [26, 27]. The gene framework, intron/exon boundaries and amino acid sequence are highly conserved among mammalian species [28]. The leptin gene itself is considered a potential quantitative trait loci (QTL), impressing many production traits in cattle. The SNPs detected by Sau3AI is situated in the second intron and resulted in amino acid change at position 2059 of the protein chain (cytosine, C to thymine, T).

Leptin hormone is linked with reproduction. For example, *Lepob/Lepob* mice express a truncated form of the protein and are bothobese and infertile, but can have their fertility restored through exogenous leptin treatment [29]. Leptin receptors are present in the ventromedial and arcuate nuclei of the hypothalamus and the anterior pituitary gland, and leptin treatment both in vitro and in vivo can increase gonadotropin secretion as demonstrated in rodents and pigs [19]. In cattle and sheep, a similar effecton gonadotropins is only observed when animals havebeen pre-exposed to a severe state of under nutrition [30]. Leptin receptors are also presentin the ovary where leptin can regulate steroidogenesis [31] and improve the ability of the oocyte tosustain subsequent embryonic development [32]. Because leptin concentrations are strongly affected by adipose tissue mass, leptin is thought to be a key signalingmolecule fasten nutritional status to reproductive function [19,30]. An appropriate amount of circulating leptin appears needful for the attainment of puberty [29], and leptin concentrations in cattle increasein the period preceding puberty [33]. Long time administration of leptin hormone to beef heifers does not

succeed, however, to induce puberty at an earlier age [30]. In dairy animals, leptin concentrations augmented during all pregnancy period but start to decreaseshortly before calving to reach a nadir in early lactation [34].

Because the role of LEPon reproductive hormones GnRH/LHand since the available information regarding the effect of polymorphism of LEPgene on buffalo reproduction aspect and (*hypothalamic-pituitary-gonadal axis*) is inadequate, therefore, the existing study was carried out to identify polymorphisms in buffalo LEP gene intron II and their possible association with reproductive status of Egyptian buffaloes.

Materials and Methods

All protocols for sample collections, phenotypic observations as well as ultrasono graphic in vestigations were approved by the Egyptian Committee of Ethics at National Research Center.

Animals and blood sampling

Thisexperimental study was performed on a total number of 150 Egyptian buffaloes during its admission to veterinary clinicsubjected to ultrasonographic examination for diagnosis of any reproductive disorders during follow up in veterinary clinics of Qaluobia governorate. Five ml blood specimens were collected from all animals through the jugular vein using vacutainer tubes coated with EDTA as anticoagulant. The blood samples were stored at -20 ℃in deep freeze as far as molecular geneticstudies are performed.

DNA isolation

High quality, whole genomic DNA was isolated from previously preserved blood samples using Reliaprep TM DNA blood kit (Promega, USA) according to the manufacturer's protocol. The total DNA concentration and purity were estimated by determining the absorbance at 260 and 280 nm using UV-visible range spectrophotometer according to the method described by Sambrook and Russell [35]and aliquoted to 50 ng/µL before PCR amplification. All the DNA samples had 260/280 optical density (OD) ratios in the range of 1.8 to 2, indicating high purity. DNA samples were stored at -20 ℃ until used for PCR assay.

PCR amplification

The genomic bovine leptin sequences which consist of three exons was obtained from Gene Bank (accession No. U50365). Sequence of primers and PCR reaction described by Liefers et al. [36]. Forward (5'-TGGAGTGGCTTGTTATTTTCTTCT-3') and reverse (5'-GTCCCCGCTTCTGGCT ACCTAACT-3') primers were used for the amplification of 422 bpfragment of intron II (closer to the polymorphic site). The PCR reaction mixture (total of 25.0 µL) contained 50 ng of genomic DNA, 2.5 µL of 10x PCR buffer, 1.5 µL of $MgCl_2$ (2.5 mmol L^{-1}), 5 pmol μL^{-1} of each primer (2.5 mmol L), 2.5 µL of dNTP (2 mrmol L^{-1}), 0.125 µL of Taq DNA polymerase (5 U μL^{-1}). PCR conditions were at 95°C for 5 min followed by 30 cycles of 94°C for 30 sec, 62°C for 40 sec and 72°C for 40 sec. After 30 cycles, reactions were completed by an extension at 72°C for 7 min. Aliquots of 10 µl of the PCR amplicons were electrophoresed using 2% Ethidium Bromide agarose gel at constant voltage of 100 V for 30 min. then visualizing under UV light with a Gel Doc 1000 system (Bio-Rad).

Leptin gene polymorphism

The PCR products were then resolved by SSCP analysis after optimization of non-denaturing PAGE concentration and other running conditions. The SSCP was performed on a total volume of 20 µl from which 10 µl PCR products were mixed with 10µL of denaturing solution (98% formamide, 10 mM NaOH, 20 mM EDTA, pH 8.0,0.05% bromophenol blue, 0.05% xylene cyanole). The PCR ampliconswere denatured by heating for 10 min at 95 ℃, snap chilled on ice for 10 min and resolved on to12% polyacrylamide gel [29:1 acrylamide:bisacrylamide, 10 mLTBE buffer (Trisbase, Boric acid, Na2EDTA), 2.5 mL glycerol,17.5 mL deionized water, 400 µ lammonium per sulfate and 40µl of TEMED]. Electrophoresis was performed using 1x TBE buffer, 160 V for14–16 h/din a Protean II xi vertical electrophoresis unit (BioRad, USA). Gel was stained using silver staining protocol to visualize DNA-fragments onpolyacrylamide gels according to Sanguinetti et al. [37] with some modification [38]. The DNA samples showing different patterns on SSCP gels were selected and PCR amplified for sequencing. At least two representative DNA samples for each SSCP variant were utilized for DNA sequencing.

DNA sequencing and bioinformatics analysis

About 20µL (50 ng/µl) of purified PCR amplicons (using QIA quick gel extraction kit. QIAGEN, Germany) of different electrophoresis patterns were sequenced in both directions using 20µl (5 pmol/µl) (5pM), forward and

reverse primers. The sequencing runs were performed on 3730xl DNA Analyzer (Applied Biosystems™ | Thermofisher) using polymer POP7. The sequencing chemistry is BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™). The sequences obtained from the normal automated DNA sequencer were analyzed using the NCBI BLASTn on line software tool after converting sequences into FASTA format (http://www.ncbi.nlm.nih.gov), and BioEdit Sequence Alignment Editor [39].

Statistical analysis

The correlation between polymorphic patterns of LEP gene and fertility status was determined by X^2-test using SPSS version 18 computer software [40].

Results

Genotyping of the leptin gene

The prime sequence used for PCR amplification of (intron II) fragment between exon IIand exon III of Egyptian buffalo. A fragment of 422 bp (Fig. 1) was successfully amplified using the designed primer pair. Using SSCP on the PCR amplicons revealed two different patterns on the gel represented the two variants A and B which are forming the twogenotypes AA represented by three bands and BB represented by two band on denaturing polyacrylamide gel (Fig. 2). The most frequent genotype in fertile buffalo was AA represented by 64%, while its distribution among infertile one is 36% distributed as 7%, 11% and 17% in buffalo with abnormal ovary, abnormal uterus and with both abnormal ovary and uterus respectively (Table 1). Concerning the genotype BB, Table one shows that genotype BB is equally distributed by 50% in both fertile and infertile animals. Among the infertile buffalo, genotype BB distributions were 5%, 9% and 12% in animals with abnormal ovary, abnormal uterus and abnormal ovary and uterus respectively. Moreover, table one pointed out that, the frequency of allele A was non significantly higher (0.7) in fertile than in infertile (0.57) animals, while its frequency in buffalo with abnormal ovary and uterus (17.3) was significantly higher than (7.1) others with abnormal ovary only. The frequency of allele B was (0.29) and (0.43) in fertile and infertile buffalo respectively (Table 1). While it was nearly equal in the 3 categories of infertile buffalo (Table 1).

Sequence analysis

Results of DNA sequencing of PCR amplicons with different electrophoretic patterns by SSCP were subjected to BLASTn analysis to ascertain their identity as leptin gene. Figure 3 pointed out that, the sequence alignment of our normal sequence with *Bubalusbubalis* leptin gene retrieved from NCBI database (Gen Bank Acc. No. AH013754.2) showing 100% identities. A fragment of 422bp of the amplified regions of normal and polymorphic leptin gene of studied population were sequenced in both forward and reverse directions and aligned using BLASTn to confirm the presence of polymorphisms. Alignment of 367 out of 422bp of sequenced PCR amplicon of both normal and polymorphic one shows a seven point mutations, three of them were (A-G,T-Cand C-T) transition and four transversion mutations (three C-G and one C-A).

TABLE 1. Genotype and allele frequencies of leptin gene in examined Egyptian buffaloes.

Pattern (Genotype)	Fertile animals No (%)	Infertile animals				Total No (%)
		Abnormal ovary	Abnormal uterus	Abnormal ovary and uterus	Total	
Pattern I AA	63 (64%)	7 (7.1%)	11 (11.2%)	17 (17.3%)	35 (36%)	98 65%
Pattern II BB	26 (50 %)	5 (10%)	9 (17%)	12 (23%)	26 (50 %)	52 35 %
A	126 f = 0.70	14 f = 0.58	22 f = 0.55	34 f = 0.58	70 f = 0.57	196 f = 0.65
B	52 f = 0.29	10 f = 0.41	18 f = 0.45	24 f = 0.41	52 f = 0.43	104 f = 0.35
Total	89 (59%)				61 (41 %)	150 100 %

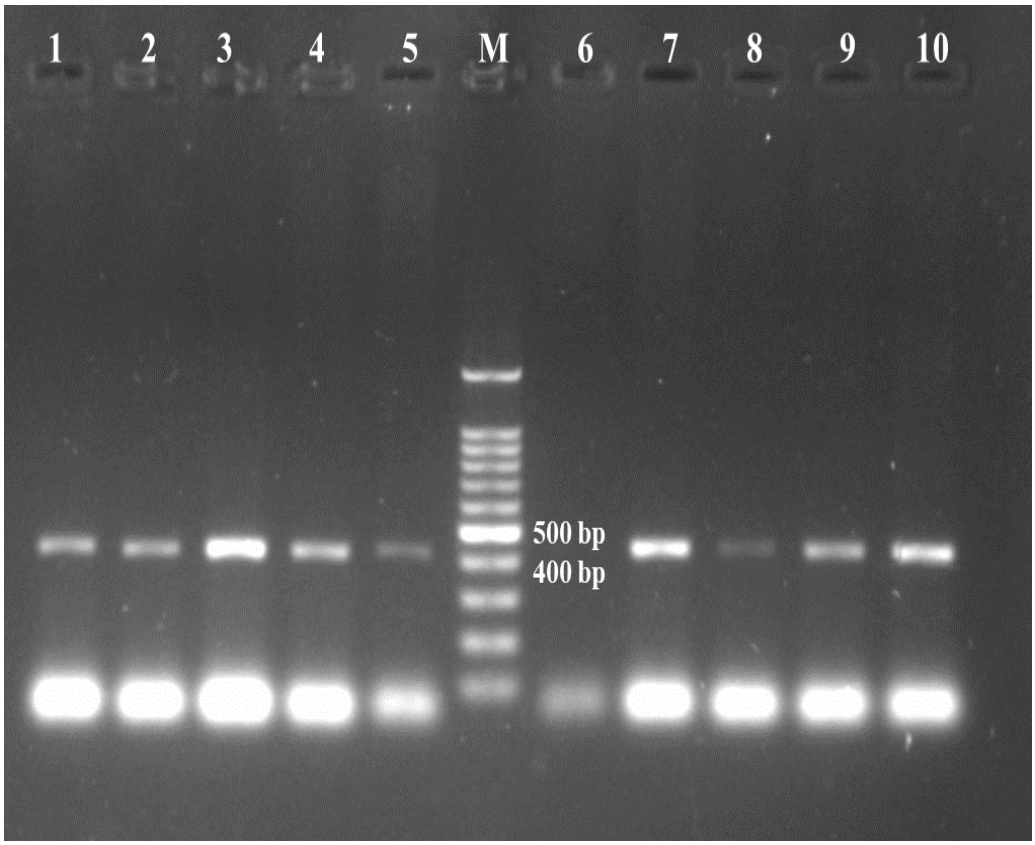

Fig. 1. Agarose gel electrophoresis of PCR amplicons of leptin gene (intron 2) in Egyptian buffalo. Lane M represent 100 bp molecular weight DNA ladder, lane from 1-5 and from 7 – 10 represent 422 bp PCR amplicons of leptin gene while lane 6 represent negative PCR reaction.

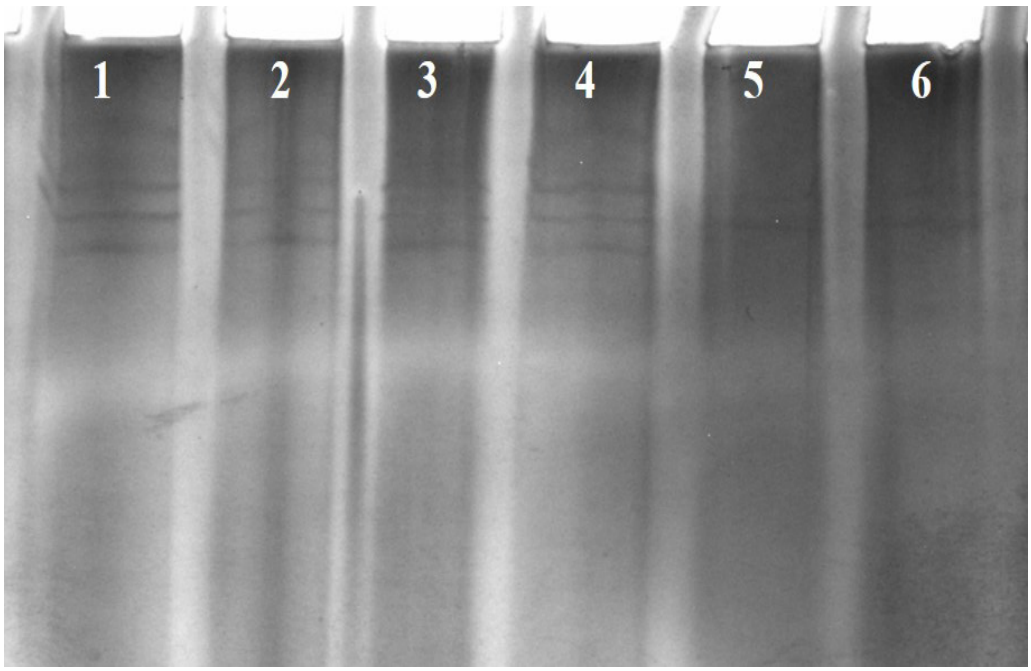

Fig.2. Non-denaturing polyacrylamide gel electrophoresis, showing different single-strand conformation polymorphism (SSCP) variants of leptin gene intron 2 (lane 1- 4 variant A, lanes 5 - 6 variant B).

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Query  1     CTGGGTCTTCTAAATTGCAGGCAGATTCTTTACCATCTGAGCCACCAGGGAAACCCATAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3017  CTGGGTCTTCTAAATTGCAGGCAGATTCTTTACCATCTGAGCCACCAGGGAAACCCATAA  3076

Query  61    GAACTTGTGAAGACTATTAAGATAGTCATTTAGACAACAAGACTATCTTAATAGTCTTCA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3077  GAACTTGTGAAGACTATTAAGATAGTCATTTAGACAACAAGACTATCTTAATAGTCTTCA  3136

Query  121   TAAGGTCTTCATGAGACTAAATTAGATAAAGCAAGTGACCCTCCCTGCATACCCTTGCAG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3137  TAAGGTCTTCATGAGACTAAATTAGATAAAGCAAGTGACCCTCCCTGCATACCCTTGCAG  3196

Query  181   AACCAGAACTGTGTGTGCCCTCTTTCAAGGTTTTCAGTCATGACTTTTGATAGCTTCCCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3197  AACCAGAACTGTGTGTGCCCTCTTTCAAGGTTTTCAGTCATGACTTTTGATAGCTTCCCA  3256

Query  241   CCTTAAAATCCAACTTGCTCACCTGCGTGGAGCAATCTGGAGACTTCCACATCTCCTGAC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3257  CCTTAAAATCCAACTTGCTCACCTGCGTGGAGCAATCTGGAGACTTCCACATCTCCTGAC  3316

Query  301   CACTCTATATTTCTAACAGTGGCTTTGGGAAGCCAGGGAGCAGTTAGGTAGCCAGAAGCG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3317  CACTCTATATTTCTAACAGTGGCTTTGGGAAGCCAGGGAGCAGTTAGGTAGCCAGAAGCG  3376

Query  361   GGGAC  365
             |||||
Sbjct  3377  GGGAC  3381
```

Fig. 3. Sequence alignment of 365 nucleotide segment of Leptin geneof Egyptian buffalo with Bubalus bubalis leptin gene intron 2 (GenBank Acc. No. AH013754.2), showing 100% identities.

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Patt I   1   CCTGGGTCTTCTAAATTGCAGGCAGATTCTTTACCATCTGAGCCACCAGGGAAACCCATA 60
Patt II  1   ............................................................ 60

Patt I   61  AGAACTTGTGAAGACTATTAAGATAGTCATTTAGACAACAAGACTATCTTAATAGTCTTC 120
Patt II  61  ............................................................ 120

Patt I   121 ATAAGGTCTTCATGAGACTAAATTAGATAAAGCAAGTGACCCTCCCTGCATACCCTTGCA 180
Patt II  121 ............................................................ 180

Patt I   181 GAACCAGAACTGTGTGTGCCCTCTTTCAAGATTTTCAGTCATGACTTTTGATAGCTTCCC 240
Patt II  181 ............................G.............................. 240

Patt I   241 ACCTTAAAATCCAACTTGCTCACCTGCGTGGAGCAATCTGGAGACTTCCATATCTCCTGA 300
Patt II  241 ............................................C......... 300

Patt I   301 CCACTCTATATTTCTAACAGTGGCTTTGGGAAGCCAGGGAGCAGCTAGGTACCCCCAACC 360
Patt II  301 ..........................................T......G..AG..G. 360

Patt I   361 GGGGACA 367
Patt II  361 ....... 367
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Fig. 4. Pairwise sequence alignment of normal sequence of Egyptian buffalo leptin gene (intron 2) and polymorphic one using BioEdit, shows seven point mutations, three of them were transition (A-G, T-C and C-T) and four transversion mutations (three C-G and one C-A). as SNPs in the pattern II.

The positions of these mutation were illustrated in (Fig. 4). Our data was submitted to gene bank.

Discussion

SSCP analysis is a simple and sensitive technique for detection of mutation and genotyping of organisms. The principle of this technique is based on the real fact that single-stranded DNA has a defined configuration. Altered of this con as a result of insertion, deletion or substitution of a single base leading to change in the DNA sequence which can cause single-stranded DNA to migrate differently (variation in electrophoretic mobility patterns) under nondenaturing electrophoresis conditions. Subsequently wild-type and mutant DNA samples display (mobility shift) different band patterns [41, 42]. In the present study, PCR-SSCP analysis of 422 bp fragment of leptin gene (intron 2) from150 buffaloes, revealed 2 types of variants. All the 2 SSCP variants (A, B) were observed with different ratios in examinedbuffaloes. On the basis of the Hardy-Weinberg formulas, thefrequencies ofgenotype AA was found to be highest with 64%, and 36% in fertile and infertile animals respectively. While genotype BB was equally distributed among fertile and infertile buffalo with 50% frequency. Regarding to the frequency of the tow alleles in the studied population, our results pointed out that, variant A frequency in fertile buffalo was 0.7 while in infertile buffalo it was 0.57. On the other hand, frequency of variant B was 0.29 and 0.43 in fertile and infertile animals respectively. Sequencing data were analyzed using BioEdit Sequence Alignment. Pairwise sequence alignment of 365 bp fragment of Egyptianleptin gene with the correspondence of Bubalus bubalis reference sequence NCBI (Gen Bank Acc. No. AH013754.2), showing 100% identities. At the same time, alignment of the normal (AA) and polymorphic (BB) sequence obtained in our results pointed out that 7 different SNPs were found in BB genotype. Several SNPs have been identified within bovine leptin gene and have been associated with milk composition, calving difficulty, gestational length and perinatal mortality rate [43]. The g.1620A [G SNP (Gen-Bank: Y11369), located in intron II of the bovine leptin gene [44], has been positively correlated with weaning weight in Nelore cattle [44]. Genetic diversity has been found also in leptin gene of river buffalo breeds. Three SNPs, one in the promoter region and two in the exon 3 have been found and a polymorphic microsatellite with at least 10 alleles has been reported in the intron 1 for four buffalo breeds by Vallinoto et al. [21]. The exon III was found to be polymorphic also in Mehsana buffalo, where Jhala et al. [45] found 3 polymorphic sites, during studing of the Leptin locus for the Mediterranean buffalo breed, Orru` et al. [46] found a total of 12 SNPs from the intron 1 to the exon 3. Although the afore mentioned studies reported such great genetic diversity, no association studies have been achieved so far in order to confirm their effectiveness in reproductive traits. Tanpure et al. [47] found three SSCP variants (A, B and C) while investigating the genetic polymorphism in intron I of leptin gene in different buffalo breeds. The variant B was found to be associated to fat percentage in Mehsana breed, indicating the rule of the gene in fat milk metabolism.

Several SNPs have been reported in the coding region of the gene [47-49] found that some SNPs are associated to production, live body weight, energy balance, feed conversion rate as well as fertility in Holstein heifers.It is generally accepted that leptin may be a strong candidate gene for economically import production traits such as meet quality, feed intake and reproduction function. Passos et al. [50] described effect of leptin polymorphism on its expression on adipose tissues in beef cattle, when frequency of A allele was 0.91 and B allele was 0.09. Moreover, this polymorphism was associated with energy balance and fertility [50], milk yield [51, 52] and somatic cell count in milk [53] and reproductive efficiency [54].

Conclusions

Leptin gene intron II of examined Egyptian buffalo cowsis polymorphic with seven SNPs (point mutation). No significant association between the detected SNPs and reproductive status was recorded in the studied buffaloes. However, these results indicate that this polymorphism is selectively neutral in relation to the improvement of fertility of Egyptian buffaloes, they also add an important information in terms of genetic variability of economically important traits and identify useful genetic polymorphisms used for marker assisted selection (MAS) programs, consequently improve animal breeding and this should be considered in future studies on buffaloes.

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Conflict of interest

All authors declare that there are no conflicts of interest.

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تفاعل البلمرة المتسلسل وتحليل تتابع التسلسل لدراسة تعدد أشكال جين اللبتين وارتباطه بالحالة الإنجابية للجاموس المصري.

حازم احمد الدبيكى[1] ، كريمة غنيمى محمد محمود[1] ، خالد عبد الحميد عبد الرازق[1] ، احمد سيد احمد سوسة[1] ، محمد محمود مصطفى قنديل[2] و يوسف فوزى احمد[1]

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جين اللبتين هو أحد الجينات المهمة التى لها دور فعال في عملية التمثيل الغذائي ونمو الحيوانات. اللبتين له تأثيرات عديدة على الأنسجة والغدد الصماء التي تؤثر في النهاية على عمليات الايض الخاصة بطاقة الجسم كله والتى لها تأثير كبير على أداء وفاعلية أنواع كثيرة من الثروة الحيوانية. بسبب امتلاكه لخصائص بيولوجية أساسية ، يعد اللبتين أحد أهم مؤشرات الجينات المرشحة لدراسات انتقاء الحيوانات باسخدام برامج (MAS) .أجريت هذه الدراسة للتعرف على تعدد أشكال الجين اللبتين وعلاقته بالحالة الإنجابية في الجاموس المصري. أجريت هذه الدراسة على مجموعه مكونة من ١٥٠ من اناث الجاموس المصرى التى تم فحصها بالموجات فوق الصوتية لتتقيم القدرة الإنجابية. وقد تم تجميع عينات دم من كل هذه الحيوانات وحفظها عند درجة حرارة ٢٠-. تم عزل الحامض النووى الديؤكسى ريبوز (DNA) من عينات الدم. تم تحديد وتكثير جزء من جين اللبتين بمقدار ٤٢٢ نيوكليوتيدة من عينات الحمض النووي المعزولة باستخام بادئ خاص لهذا الجزء وذلك باستخدام جهاز تفاعل البلمرة المتسلسل وتم إجراء تقنية PCR-SSCP على الجزء المكثر لتحديد الطفرات التى قد تحدث فى الجين. عينات تفاعل البلمرة المتسلسل الموجبة و التى لها نمط مختلف باستخدام الفصل الكهربى تم تنقيتها واجراء تحليل تتابع التسلسل لها في كلا الاتجاهين باستخدام البادئ الامامى والخلفى. كما تم تحليل نتائج تتابع التسلسل باستخدام برامج NCBI BLASTn و مضاهات التسلسل BioEdit. أظهرت النتائج اكتشاف نمطين من جين البتين هما (AA و BB). كان النمط الوراثي الأكثر شيوعًا في الجاموس عالى الخصوبة هو AA الذي يمثل ٦٤٪ ، في حين أن توزيع هذا النمط بين الجاموس العقيم كان ٣٦٪. فيما يتعلق بالنمط الجيني BB ، أوضحت النتائج أن النمط BB موزعة بالتساوي بنسبة ٥٠ ٪ في كل من الحيوانات عالية الخصوبة والاخرى التى تعانى من مشاكل ضعف القدرة الانجابية. أشارت نتائج تسلسل الحمض النووي لجين اللبتين الطبيعى مع مثيلتها من النمط المتعدد الأشكال الى وجود عدة طفرات، و لكن لم تسجل النتائج الاحصائية وجود أي ارتباط كبير بين هذه الطفرات (SNPs) والحالة الإنجابية في الجاموس.