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Single Nucleotide Polymorphisms of GnRHR Gene and Its Relationship with Reproductive Performance in Egyptian Buffaloes

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THIS WORK aimed to search for single nucleotide polymorphisms of gonadotropin releasing hormone receptor (GnRHR) gene in Egyptian buffalo which involved in endocrine control of fertility. An ultrasonographic examinations of anestrum and repeat breeders' buffaloes were performed prior to blood sampling through a transrectal ultrasonography. DNA was extracted from blood and the PCR and single-strand conformation polymorphism (SSCP) techniques were adopted to study of GnRHR gene polymorphism. The PCR amplified a fragment with 240-bp in size and the SSCP results showed that there is a genetic polymorphism with three different patterns (AA, BB and CC) in Egyptian buffalo. The CC genotype was associated with smooth inactive ovary, while BB genotype was associated with fertility in buffalo. Multiple sequence alignment of the three patterns sequences revealed that GnRHR had 5 single nucleotide polymorphisms including one nucleotide insertion, one nucleotide deletion and 3 nucleotide substitutions. Insertion was at position 4 in pattern III. The deletion was at position 189 in pattern III, while the 3 nucleotide substitutions were at positions 204 (T/G), 206 (T/A) and 207 (A/T). In conclusion, GnRHR gene could be used as a candidate marker for fertility in Egyptian buffaloes with its mutation is related to ovarian inactivity.

Keywords: Buffalo, GnRHR Gene, Nucleotide sequences, SSCP analysis.

Gonadotropin-releasing hormone is the key that plays important roles in vertebrate reproduction (Krieger *et al.*, 1982). Its function is to stimulate the synthesis and release of pituitary gonadotropins (follicle stimulating hormone and luteinizing hormone) which regulate gametogenesis and steroidogenesis at gonadal level (Okuzawa and Kobayashi, 1999).

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The interaction of GnRH with its receptor is important in the endocrine control of reproduction. The response of pituitary gonadotropes to GnRH correlates with the concentration of GnRH receptors on the cell surface (Wise *et al.,* 1984). Mutations in GnRHR gene were found to be responsible for many reproductive genetic disorders in humans (Rosenthal *et al.,* 1994) such as hypogonadotropic hypogonadism during puberty (Kottler *et al.,* 1999).

The GnRHR gene is a good candidate gene for mutation analysis associated with reproductive performance**.** The polymorphism in promoter region of GnRHR had been associated with transmitting ability for days to first service in cattle (Derecka *et al.,* 2010). Moreover, Yang *et al.* (2011) reported that A- or C alleles had favorable positive effects on sperm quality traits in bulls. In bovine, Liron *et al.* (2011) reported that European *Bostaurus* had lower frequencies of the C allele than *B. indicus*, while Creole and Wagyu breeds had intermediate frequency.

Molecular basis controlling physiological pathways related to infertility in buffalo still unknown. Therefore, the current study was aimed to detect the genetic polymorphism of GnRHR gene and its relation to fertility in Egyptian buffalo.

Material and Methods

Animals

A total numbers of 186 female Egyptian buffaloes belonged to Meet Kenana village, Qaluobia with the history of anestrum (n=42), repeat breeding (n=28) and fertile (n= 116) were used in this study during the year of 2013. Animals were admitted to the veterinary clinic with owner complains were infertility problems treatment or pregnancy diagnosis. Case history of each animal was recorded. Rectal and ultrasonographic examinations were accomplished once for three successive weeks to define the animal reproductive status and/or disorder.

Blood sampling and DNA isolation

Blood samples were taken before gynecological examination from jugular vein with vacutainer tubes containing EDTA as anticoagulant. Genomic DNA was extracted with the QIAamp DNA blood kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions.

PCR Reaction and DNA Amplification

Polymorphism was evaluated using primers designed for Egyptian buffalo GnRHR gene. The primers used in this study for amplification of 240-bp fragments of GnRHR gene were those described by Milazotto *et al.*(2008) with the following nucleotide sequence:
F: AAACTACAACTGAATCAGTC
R: TAGAGAGAAATATCCATATA

Amplification reactions were done in 50 µl volume, containing 5.00µl buffer 10x, 1.00µl of 2.5 mM (dNTPs mixture), 0.30µl Taq polymerase (5U/µl), 0.25µl primer, 3.00µl of 25 mM ($MgCL_2$), 35.20µl water (nuclease free water) and 5.00µl DNA sample. The PCR conditions were: one cycle at 95 °C for 4 min, and 35 cycles of the sequence: 95 °C for 60 sec., 55 °C for 30 sec. and 72 °C for 60 sec. Upon completion of the reaction, the products were subjected to electrophoresis in 2% agarose gel at 60 V for 2 hrs. Visualization of the bands was done under ultraviolet Trans-illumination and a photo was taken in Bio-Rad Gel-Doc. System. The amplified product size was compared with the 100bp Ladder DNA marker.

Single strand conformation polymorphism (SSCP)

The SSCP was used to screen the mutations in the amplified segment. PCR products (7.00µl) were mixed with 8.00µlof denaturing solution (98% formamide;20mM EDTA, pH 8.0; 0.05%bromophenol blue; 0.05%xylene cyanol). After that, the samples were denatured by heating at 95 °C for 8 min., then they were put on ice for 8 min. and loaded in 1x TBE buffer on to12% polyacrylamide gel consists of [29:1 acrylamide: bisacrylamide, 10 ml 1xTBE buffer (Trisbase, Boric acid, Na_2EDTA), 2.5 ml glycerol, 17.5 ml deionized water, 400µl APS solution(ammonium per sulfate) and 40µl of TEMED (N, N, N´, N´-Tetramethylethylenediamine].Electrophoresis was carried out at 4 °C, 90V for 18h.Silver staining technique was used to visualize the separated DNA-fragments with high sensitivity on polyacrylamide gels according to Sanguinetti *et al.*(1994) with some modification (Benbouza *et al.,* 2006).

Sequence analysis

For genetic sequencing, the PCR product was refined by QIAquick PCR purification kit (QIAGEN). The PCR products giving unique SSCP band patterns were analyzed by direct sequencing by Macrogen Incorporation (Seoul, South Korea). Sequence data were analyzed and alignment was carried out using NCBI/BLAST/blastn suite. Sequence data were further analyzed for detecting single nucleotide polymorphism using BioEdit software.

Results and Discussion

Normal reproductive function necessitates the precise temporal and quantitative regulation of hormone excretion at all levels of the hypothalamic–pituitary–gonadal axis. Failure of the normal pattern of GnRH gene leads to clinical disorder of idiopathic hypogonadotropic hypogonadism and infertility. In the present study, PCR-SSCP technique was used to study the polymorphism of GnRHR gene in female Egyptian buffaloes. Primers used in this study was a flanked 240-bp fragment of Egyptian buffaloes GnRHR gene (Fig.1). The gene locus of GnRHR was found to be polymorphic with three different SSCP patterns in buffaloes (Fig.2). Pattern I (AA),II (BB) and III (CC) entailed four, five and six bands, respectively. Likewise, Kerekoppa *et al.*(2015) verified high genetic variation in GnRHR gene in cattle using PCR-SSCP technique, where fragment 1and 3 showed two patterns, fragment 2 and 4 exhibited three patterns and fragment 5 was monomorphic in bulls.

The DNA sequence of 207-bp fragment of buffaloes GnRHR gene out of the amplified 240-bp was determined and sequence alignment with published sequence of Egyptian buffaloes (Bubalus bubalis) gonadotropin-releasing hormone receptor, mRNA sequence (accession number:NM_001290857.1) was carried out using BLAST. The 207bp segment possessed99 % identities (Fig.3).Current study results also revealed that the sequence of the same DNA segment was 99 % identities with the accession number: GenBank: gbEU621854.1 (Fig.4). Multiple sequence alignment of the three patterns sequences (Fig.5) revealed that GnRHR gene had five SNPs (single nucleotide polymorphisms) including one nucleotide insertion, one nucleotide deletion and three nucleotides substitution. In pattern III, insertion was at position 4, deletion was at position 189, and the 3 nucleotides substitutions were at positions 204 (T/G), 206 (T/A) and 207 (A/T). In this respect, Kerekoppa *et al.* (2015) reported high degree of genetic variation (14 SNPs) in the coding region of GnRHR gene among Holstein Friesian, Jersey, Malnad Gidda, and Deoni cattle. Also, Lirón *et al.* (2011) characterized bovine GNRHR polymorphisms in beef bulls and found eight SNPs of which five in the coding regions, might at least part responsible for the differences within and among beef breeds in the time when bulls reach puberty.Li *et al.* (2011) showed that the polymorphisms detected by PCR-SSCP and DNA sequencing methods of the GnRHR gene were significantly associated with litter size in Shaanan and Boer goats.

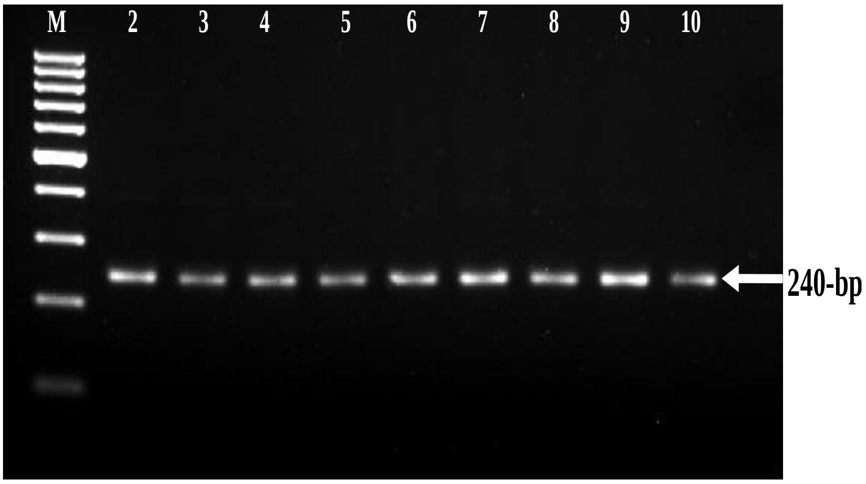


Fig. 1. Agarose gel stained with ethidium bromide showing the PCR product of buffalo GnRHR gene. M: 100-bp ladder. Lanes 2-10 :240-bp PCR product of GnRHR gene.

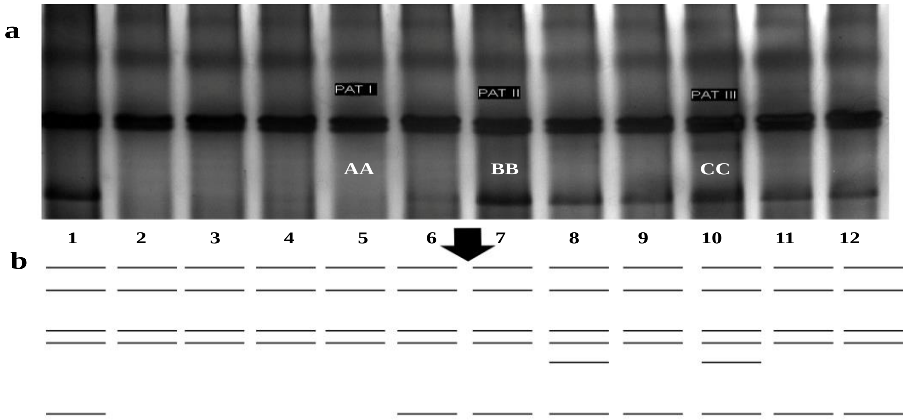
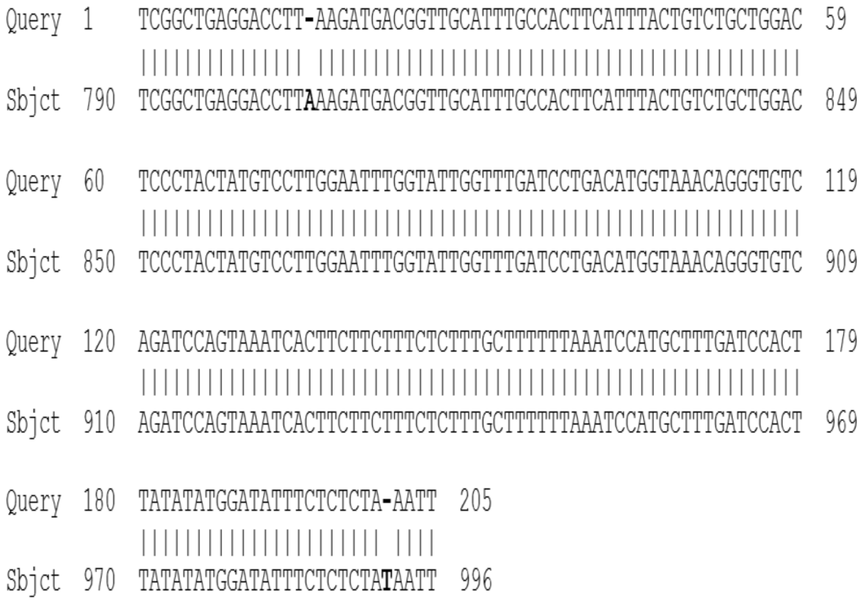


Fig. 2. a. Three different SSCP patterns of GnRHR gene in tested Egyptian buffalo on 12 % silver stained-polyacrylamide gel. Lanes : 2-3-4-5: pattern I (AA). Lanes : 1- 6-7-9-11-12: pattern II (BB). Lanes : 8-10: pattern III (CC). b) Diagram showing SSCP banding patterns of 240 bp fragment of GnRHR gene of Egyptian buffaloes.



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Query  1    TCGGCTGAGGACCTT-AAGATGACGGTTGCATTTGCCACTTCATTTACTGTCTGCTGGAC  59
            ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  790  TCGGCTGAGGACCTTAAAGATGACGGTTGCATTTGCCACTTCATTTACTGTCTGCTGGAC  849

Query  60   TCCCTACTATGTCCTTGGAATTTGGTATTGGTTTGATCCTGACATGGTAAACAGGGTGTC  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  850  TCCCTACTATGTCCTTGGAATTTGGTATTGGTTTGATCCTGACATGGTAAACAGGGTGTC  909

Query  120  AGATCCAGTAAATCACTTCTTCTTTCTCTTTGCTTTTTTAAATCCATGCTTTGATCCACT  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  910  AGATCCAGTAAATCACTTCTTCTTTCTCTTTGCTTTTTTAAATCCATGCTTTGATCCACT  969

Query  180  TATATATGGATATTTCTCTCTA-AATT  205
            |||||||||||||||||||||| ||||
Sbjct  970  TATATATGGATATTTCTCTCTATAATT  996
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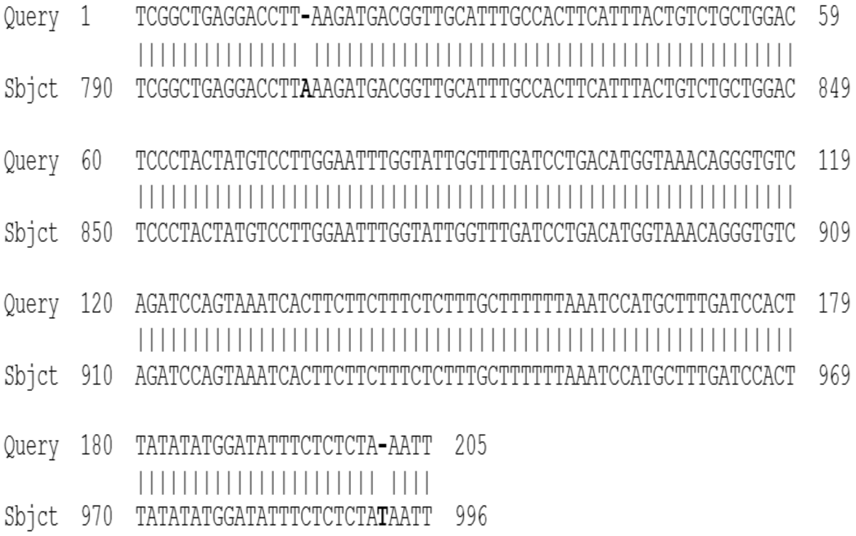
Fig. 3. Sequence analysis of 207 segment of Egyptian buffalo gonadotropin-releasing hormone receptor (GnRHR) amplified product compared to Bubalus bubalis (GnRHR). GnRHR mRNA Sequence ID: NM_001290857.1.



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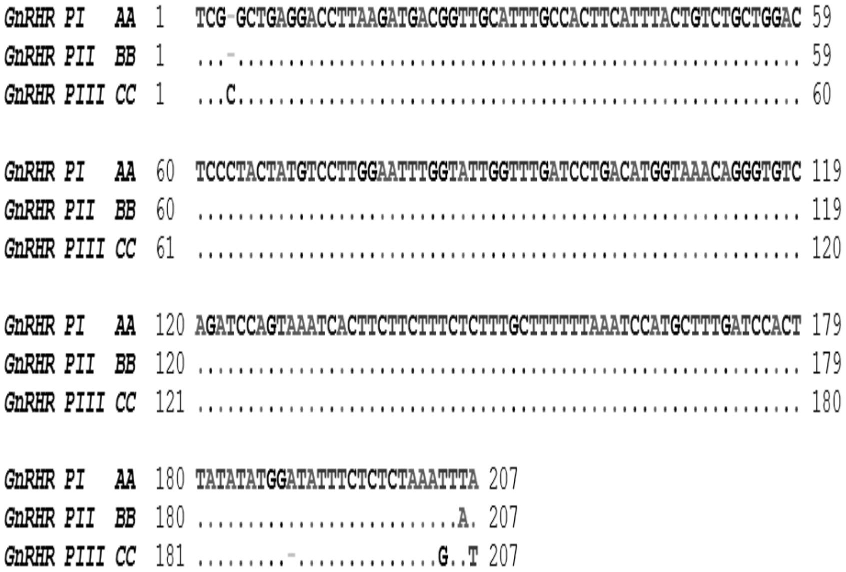
Query  1    TCGGCTGAGGACCTT-AAGATGACGGTTGCATTTGCCACTTCATTTACTGTCTGCTGGAC  59
            ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  790  TCGGCTGAGGACCTTAAAGATGACGGTTGCATTTGCCACTTCATTTACTGTCTGCTGGAC  849

Query  60   TCCCTACTATGTCCTTGGAATTTGGTATTGGTTTGATCCTGACATGGTAAACAGGGTGTC  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  850  TCCCTACTATGTCCTTGGAATTTGGTATTGGTTTGATCCTGACATGGTAAACAGGGTGTC  909

Query  120  AGATCCAGTAAATCACTTCTTCTTTCTCTTTGCTTTTTTAAATCCATGCTTTGATCCACT  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  910  AGATCCAGTAAATCACTTCTTCTTTCTCTTTGCTTTTTTAAATCCATGCTTTGATCCACT  969

Query  180  TATATATGGATATTTCTCTCTA-AATT  205
            |||||||||||||||||||||| ||||
Sbjct  970  TATATATGGATATTTCTCTCTATAATT  996
```

Fig. 4. Sequence analysis of 207 segment of Egyptian buffalo GnRHR amplified product compared with Bubalus bubalis pituitary type-I GnRH mRNA, complete cds Sequence ID: EU621854.1..



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GnRHR PI   AA 1   TCG-GCTGAGGACCTTAAGATGACGGTTGCATTTGCCACTTCATTTACTGTCTGCTGGAC 59
GnRHR PII  BB 1   ...-........................................................ 59
GnRHR PIII CC 1   ...C........................................................ 60

GnRHR PI   AA 60  TCCCTACTATGTCCTTGGAATTTGGTATTGGTTTGATCCTGACATGGTAAACAGGGTGTC 119
GnRHR PII  BB 60  ............................................................ 119
GnRHR PIII CC 61  ............................................................ 120

GnRHR PI   AA 120 AGATCCAGTAAATCACTTCTTCTTTCTCTTTGCTTTTTTAAATCCATGCTTTGATCCACT 179
GnRHR PII  BB 120 ............................................................ 179
GnRHR PIII CC 121 ............................................................ 180

GnRHR PI   AA 180 TATATATGGATATTTCTCTCTAAATTTA 207
GnRHR PII  BB 180 ..........................A. 207
GnRHR PIII CC 181 .........-..............G..T 207
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Fig. 5. Multiple sequence alignment of 3 different patterns of GnRHR gene of Egyptian buffaloes by BioEdit showed 5 SNPs.

In the current study, allele-frequency analysis of genetic polymorphism patterns of GnRHR gene locus in investigated buffaloes (Table 1) revealed that pattern I (AA) was the most encountered compared with patterns II (BB) and III (CC) (51.7% vs. 44.1% and 4.3%, respectively).Similarly, Liron *et al.* (2011) noticed lower frequencies of the C allele in European Bostaurus than B. indicus, meanwhile intermediate frequency of GnRHR alleles was recorded in Creole and Wagyu breeds.

TABLE 1. Patterns and frequencies of gonadotropin releasing hormone receptors gene polymorphism in fertile and infertile Egyptian buffaloes.

Pattern		**Fertile animals**	**Infertile animals**				**Total**
			Anestrum		**Repeat breeder (Abnormal Uterus)**	**Total**	
			Abnormal ovary	**Abnormal ovary and uterus**			
Pattern I (AA)	n	56	8	18	14	40	**96**
	%	58.33 %	8.33 %	18.75 %	14.58 %	41.66 %	51.62 %
Pattern II (BB)	n	60	6	2	14	22	82
	%	73.17 %	7.31 %	2.43 %	17.07 %	26.82 %	44.08 %
Pattern III (CC)	n	0	8	0	0	8	8
	%	0	100 %	0	0	100 %	4.30 %
Total	n	116	22	20	28	70	186
	%	62.4 %	-	-	-	37.6 %	100 %

The ultrasound findings were used as the phenotype information to classify the animals into fertile and infertile animals. Accordingly, 116 animals were considered as fertile and represented about 62.4 %.On the other hand, 70 animals were found to suffer from infertility and those represent 37.6 % of total tested animals. Pattern I (AA) was seen in 96 animals, with an incidence of 58.33% and 41.66 % in fertile and infertile animals, respectively. Pattern II (BB) appeared in 82 animals with a frequency of 73.2% and 26.8% in fertile and infertile buffaloes, respectively. Pattern III (CC) was displayed in 8 animals with an incidence of 100% in infertile animals suffered from smooth inactive ovary. These results suggested the precision of the predictability of the phenotype of animals fertility with approximately 58.33%,73.2% and 100 %accuracy for pattern I, II and III, respectively, with the latter was associated with low fertility phenotype. GnRHR mutations have been found to decrease GnRHR binding and/or activation of inositol triphosphate or phospholipase C and are related to idiopathic hypogonadotropic hypogonadism or Kallmann's syndrome in humans (Tamaya, 2002). It is possible that the mutations in GnRHR gene influence sex hormone levels which significantly impact on reproduction (Sun *et al.*, 2008). In addition, naturally occurring mutations in GnRHR was demonstrated to be responsible for human genetic disorders (Kottler *et al.,* 2000). Moreover, Sedlmeyer *et al.* (2005)found three SNPs associated with late pubertal development, another SNP associated with late menarche, and one rare haplotype associated with early age of menarche.

Conclusion

GnRHR gene in the Egyptian buffaloes is polymorphic with three different SSCP patterns (AA, BB and CC). The CC genotype was associated with ovarian disorders and BB genotype was associated with fertility in buffalo**.** These results suggest that GnRHR gene could be implied as a candidate marker for fertility in Egyptian buffaloes.

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تعدد الطرز الوراثية للنيوكليتدات الأحادية لجين مستقبلات الهرمون المحفز لهرمونات الغدة النخامية (Gn RHR) و ارتباطه بالكفاءة التناسلية للجاموس المصرى

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يهدف البحث إلى دراسة الطرز الجينية المتعددة فى النيوكلوتيدات الأحادية فى جين مستقبلات الهرمون المحفز لهرمونات الغدة النخامية (Gn RHR) و المرتبطة بالخصوبة فى الجاموس المصرى. و قد تم الفحص باستخدام الأشعة فوق الصوتية للجاموس الذى يعانى من عدم حدوث الشبق و تكرار الشياع. و قد تم أخذ عينات دم من هذه الحالات للاستخلاص الحمض النووى الديوكسى ريبوزى و استخدام تقنية تفاعل البلمرة المتسلسل (PCR) و شكل التنوع فى شريط الحمض النووى الريبوزى الأحادى الخاصة بمستقبلات الهرمون المحفز لهرمونات الغدة النخامية وطرزه الوراثية و قد نتج فى تفاعل البلمرة المتسلسل قطعة بحجم 240 bp واظهر التنوع فى النيوكلوتيدات الأحادية ثلاث طرز (AA,BB, CC) فى الجاموس المصرى. و قد وجد أن الطرز (CC) له علاقة بخمول المبايض و أن (BB) له علاقة بالخصوبة فى الجاموس. و أظهر تحليل التتابع النيوكلوتيدى للثلاث أنماط وجود تنوع فى خمسة نيوكلوتيدات و تشمل واحد إضافة و أخرى حذف و ثلاثة إحلال. و كانت الإضافة عند موضع ٤ فى النمط الثالث. و كانت الحذف عند الموضع ١٨٩ فى النمط الثالث بينما كان الإحلال عند الموضع ٢٠٤, ٢٠٦, ٢٠٧. و الخلاصة أن جين Gn RHR يمكن استخدامه كمؤشر للخصوبة فى الجاموس المصرى و أن طفراته لها علاقة بخمول المبايض.