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RFL polymorphism of three fertility genes in Egyptian buffalo

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Abstract

There is a considerable interest in the application of molecular genetics technologies in the form of specific DNA markers that are associated with various productivity traits to promote more efficient and relatively easy selection and breeding of farm animals with an advantage for inheritable traits of production and reproduction.

River buffalo is a species of great economic potential, especially in developing countries; it is the main source of meat and milk in Egypt. This study aimed to detect the genetic polymorphism of three genes; follicle-stimulating hormone receptor (*FSHR*), luteinizing hormone receptor (*LHR*) and estrogen receptor-α (*ERα*) genes which are considered candidate genes for fertility and reproduction performances. The genetic polymorphism detection of these genes in Egyptian buffaloes will enable us to identify and subsequently select the animals with high fertility performances- depending on these molecular markers- for breeding programs.

Genomic DNA extracted from 100 healthy female buffaloes was amplified using primers that were designed from the cattle *FSHR*, *LHR* and *ERα* gene sequences. The amplified fragments of *FSHR* (306-bp), *LHR* (303-bp) and *ERα* (248-bp) were digested with *Alu*I, *Hha*I and *Bgl*I restriction enzymes, respectively. The results showed that all tested buffaloes are genotyped as **CC** for *FSHR* and **TT** for *LHR*. Regarding to the *ERα* gene, 18% of the investigated buffaloes are genotypes as heterozygous AG and the remaining animals (82%) are genotyped as GG.

To the best of our knowledge, this is the first published data on the genetic characterization and nucleotide sequences of fertility genes in Egyptian buffalo. The nucleotide sequences of Egyptian buffalo *FSHR*C, *LHR*T, *ERα*G and *ERα*A alleles were submitted to nucleotide sequences database NCBI/ Bankit/GenBank and have the accession numbers JX049145, JQ885687, JQ308795 and JQ308796, respectively.

Keywords: Buffalo, *FSHR*, *LHR*, *ERα*, PCR-RFLP, Nucleotide sequences

INTRODUCTION

River buffalo is a species of great economic potential; it is the main source of meat and milk in Egypt. Genetic improvement of buffalo productivity has been dependent on concepts of quantitative genetics of various productivity traits of economic importance to promote more efficient and relatively easy selection of Egyptian buffaloes [1].

Follicle-stimulating hormone (*FSH*) starts and maintains follicular development by binding to its specific receptor (*FSHR*) in the surface of the granulosa cells in the ovary [2]. This binding allows the activation of the *FSHR* codifying gene [3]. The existence of allelic variants in *FSH*R gene was reported in cattle [4] and [5] and these changes in the molecular structure of this gene cause desensitization of the *FSH* receptors in the cell membrane which results in a less efficient hormone signal [6].

Luteinizing hormone (*LH*) is a glycoprotein hormone of pituitary origin that regulates gonadal function, including steroidogenesis and gametogenesis. *LH* action is mediated by specific receptors that are located in the plasma membrane of specific target cells in the ovary and testis [7]. The expression of the *LH* receptor (*LHR*) in the ovary is induced by follicle-stimulating hormone, estrogen, and growth factors in granulosa cells of the preovulatory follicles. In the testis, the *LHR* is expressed in fetal Leydig cells and throughout adult life. The identification of differential signaling pathways that regulate *LHR* gene expression, as well as the elucidation of molecular mechanism(s) of receptor regulation, is of major relevance to the understanding of normal reproductive physiology and the pathology of reproductive disorders [8].

Estrogens regulate cellular activity by interacting with specifying intracellular receptor proteins. Due to the functions played in reproduction, development of the mammary gland, growth and differentiation of cells, the estrogen receptor-α (*ERα*) and its gene is considered a candidate marker for reproduction and functional traits in farm animals [9].

The objective of this study was to detect the genetic polymorphism of three genes; *FSHR*, *LHR* and *ERα*; related to the fertility trait in Egyptian buffalo using PCR-RFLP technique.

MATERIALS AND METHODS

Animals

The genomic DNA used in this study was extracted from blood samples of 100 healthy female buffaloes. They were taken from two organized stations of buffaloes. The first is Mahlet Mussa station which lies near to Sakha in Kafer El-Sheakh governorate; from it, we collected 60 blood samples. The second one is El-Gmeasa station which lies near to Tanta city in Gharbia governorate; from it, we collected 40 blood samples.

Genomic DNA extraction

Genomic DNA was extracted from the whole blood of 100 unrelated female Egyptian buffaloes according to established protocol [10] with minor modifications. Briefly, 10 ml of blood taken on EDTA were mixed with 25 ml of cold 2X Sucrose-Triton and 15 ml double distilled water. The tubes were placed on ice for 10 min and mixed by inversion several times. After centrifugation, at 5000 rpm for 15 min at 4ºC, the pellet was re-suspended by 3 ml of nucleic lysis buffer. The content was mixed with 108 µl of 20% SDS and 150 µl of Proteinase K. The tubes were placed in a water bath at 37ºC overnight.

After the incubation, the tube contents were transferred to a 15-ml polypropylene tube and 2 ml of saturated NaCl was added and shaken vigorously for 15 sec. After centrifuging at 3500 rpm for 15 min at 4ºC, the supernatant was transferred to a clean 15-ml polypropylene tube and mixed with absolute ethanol. The tubes were agitated gently to mix the liquids and a fluffy white ball of DNA was formed. The precipitated DNA was picked up using the heat sealed pasture pipette, then washed twice in 70% ethanol and exposed to air to dry completely.

The DNA was dissolved in 200 µl TE buffer in 1.5-ml Microfuge tube and kept overnight in an incubator at 37ºC. DNA concentration was determined and diluted to the working concentration of 50ng/µl, which is suitable for polymerase chain reaction using NanoDrop1000 Thermo Scientific spectrophotometer.

Polymerase chain reaction (PCR)

A PCR cocktail consisted of 1.0 µM upper and lower primers specific for each tested gene (**Table 1**) , 0.2 mM dNTPs, 10 mM Tris (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin (w/v), 0.1 % Triton X-100 and 1.25 units of Taq polymerase. The cocktail was aliquot into PCR tubes with 100 ng of buffalo DNA. The reaction was cycled for 1 min. at 94°C, 1 min at an optimized annealing temperature that was determined for each primer (**Table 1**) and 1 min. at 72°C for 30 cycles. The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide to test the amplification success.

In this study, the genetic polymorphism was analyzed using primers representing exon 10 in *FSHR*, exon 11 in *LHR* and the putative promoter of exon C in *ER*α genes. We focused on these regions because they are polymorphic regions in cattle. Genetically, cattle are closely related to buffalo and always the cattle are used as a guide for the genetic studies (especially genetic mapping and polymorphism) in buffalo.

Restriction fragment length polymorphism (RFLP)

The PCR products for the three tested genes were digested with the restriction enzyme specific for each gene (**Table 1**). The restriction mixture for each sample was prepared by adding 2.5 µl of 10restriction buffer to 10 units of the restriction enzyme and the volume was completed to 5 µl by sterile water. This restriction mixture was mixed with PCR product (~25 µl) and incubated overnight at the optimum temperature of the maximum activity for each restriction enzyme. The digested PCR products were electrophoresed on agarose or polyacrylamide gels staining with ethidium bromide to detect the different genotypes of the three tested genes.

Sequence Analysis

The PCR products for each tested gene were purified and sequenced by Macrogen Incorporation (Seoul, Korea). Sequence analysis and alignment were carried out using NCBI/BLAST/blastn suite. Results of endouclease restriction were carried out using FastPCR. The nucleotide sequences of the three tested genes in Egyptian buffalo were submitted to GenBank (NCBI, BankIt).

Table 1. The sequences and information of primers used in this study

Gene	Sequences 5′ -------- 3′	PCR conditions	PCR product size	Restriction enzyme used	References
FSHR	CTG CCT CCC TCA AGG TGC CCC TC AGT TCT TGG CTA AAT GTC TTA GGG GG	94°C 1 min 60°C 1 min 72°C 1 min	306 bp	*Alu*I	[5]
LHR	CAA ACT GAC AGT CCC CCG CTT T CCT CCG AGC ATG ACT GGA ATG GC	94°C 1 min 57°C 1 min 72°C 1 min	303 bp	*Hha*I	[5]
*ER*α	TTT GGT TAA CGA GGT GGA G TGT GAC ACA GGT GGT TTT TC	94°C 1 min 56°C 1 min 72°C 1 min	248 bp	*Bgl*I	[9]

RESULTS AND DISCUSSION

In marker-assisted selection of dairy livestock, several QTL for production and reproduction performances have been identified. A number of potential candidate genes have been identified and selected for analyses based on a known relationship with physiological or biochemical processes and reproduction traits. It is seldom reported that one particular polymorphism in a candidate gene would influence several traits of economic importance in livestock at the same time.

One of the limiting factors for quick genetic improvement in the buffalo population is poor reproduction. *FSHR*, *LHR* and *ERα* are considered candidates for the markers of reproduction in farm animals. The study aimed to detect the genetic polymorphism of these three genes which are related to the fertility trait in Egyptian buffalo using PCR-RFLP technique.

FSHR gene

The *FSHR* gene was studied in *Bos Taurus*, this gene is located on chromosome 11 and its structure is determined by 10 exons and 11 introns; the first 9 exons enclose the extracellular domain whereas exon 10 encloses the transmembrane domain [11]. Our study examined the genetic polymorphism of this exon 10 in Egyptian buffalo *FSHR* gene.

The primers used in this study flanked a 306-bp fragment from exon 10 of Egyptian buffalo *FSHR* gene. The amplified fragments obtained from all tested buffalo DNA (100 animals) were at 306-bp (**Fig. 1**).

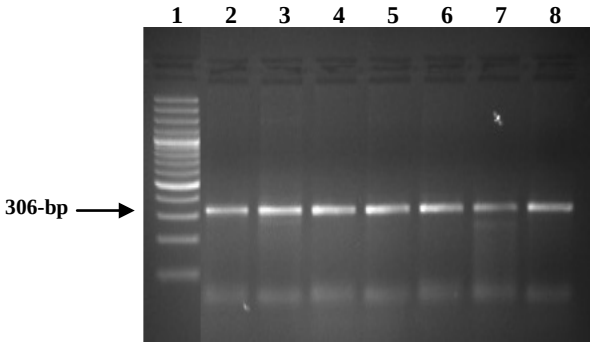

Fig. 1. Ethidium bromide-stained gel of PCR products representing amplification of *FSHR* gene in Egyptian buffalo.

Lane 1: 100-bp ladder marker
Lanes 2-8: 306-bp PCR products amplified from Egyptian buffalo DNA

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CTGCCTCCCTCAAGGTGCCCCTCATCACTGTGTCCAAGTCAAAGATCCTCCTGGTCCTGTTCTACCCCATCAACT
CCTGTGCCAACCCCTTCCTCTATGCCATCTTCACCAAGAACTTCCGCAGGGATTTCTTCATTCTGCTGAGCAAGT
TTGGCTGCTATGAAGTGCAAGCCCAGACCTATAGGTCAGAAACCTCATCCACTGCCCACAACTTTCATCCAAGG
AATGGTCACTGCCCCCCAGCTCCCAGGGTTACCAGTGGTTCCAATTACACACTTATCCCCCTAAGACATTTAGC
CAAGAACT
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Fig. 2. The sequence analysis of Egyptian buffalo *FSHR* amplified fragment

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Query  1     CTGCCTCCCTCAAGGTGCCCCTCATCACTGTGTCCAAGTCAAAGATCCTCCTGGTCCTGT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  927   CTGCCTCCCTCAAGGTGCCCCTCATCACTGTGTCCAAGTCAAAGATCCTCCTGGTCCTAT  986

Query  61    TCTACCCCATCAACTCCTGTGCCAACCCCTTCCTCTATGCCATCTTCACCAAGAACTTCC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  987   TCTACCCCATCAACTCCTGTGCCAACCCCTTCCTCTATGCCATCTTCACCAAGAACTTCC  1046

Query  121   GCAGGGATTTCTTCATTCTGCTGAGCAAGTTTGGCTGCTATGAAGTGCAAGCCCAGACCT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1047  GCAGGGATTTCTTCATTCTGCTGAGCAAGTTTGGCTGCTATGAAGTGCAAGCCCAGACCT  1106

Query  181   ATAGGTCAGAAACCTCATCCACTGCCCACAACTTTCATCCAAGGAATGGTCACTGCCCCC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1107  ATAGGTCAGAAACCTCATCCACTGCCCACAACTTTCATCCAAGGAATGGTCACTGCCCCC  1166

Query  241   CAGCTCCCAGGGTTACCAGTGGTTCCAATTACACACTTATCCCCCTAAGACATTTAGCCA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1167  CAGCTCCCAGGGTTACCAGTGGTTCCAATTACACACTTATCCCCCTAAGACATTTAGCCA  1226

Query  301   AGAACT  306
             ||||||
Sbjct  1227  AGAACT  1232
```

Fig. 3. Sequence alignment of Egyptian buffalo *FSHR* with published sequences

In order to verify the PCR product, two-way sequence analysis of the *FSHR* amplified PCR products of buffalo DNA was conducted. The buffalo amplicon obtained was found to be 306-bp (**Fig. 2**). The Egyptian buffalo *FSHR* nucleotide sequence was submitted to nucleotide sequences database NCBI/ Bankit/GenBank and has the accession number **JX049145**.

The sequence alignment of Egyptian buffalo *FSHR* with published sequence (accession number. EU148059.1, *Bubalus bubalis*) was carried out using BLAST and showed that the Egyptian buffalo *FSHR* possess identities at 99% with only one SNP (**G/A**) at position 59 (**Fig. 3**).

These PCR amplified fragments (306-bp) were digested with *Alu*I endonuclease. Depending on the presence or absence of the restriction site at position 243^244 (AG^CT), we can easily differentiate between 3 different genotypes. CC with two digested fragments at 243- an 63-bp, GG with three digested fragments at 193-, 63- and 50-bp and CG with four digested fragments at 243-, 193-, 63- and 50-bp

All buffaloes investigated in this study are genotyped as **CC** where all tested buffalo DNA amplified fragments at 306-bp were digested with *Alu*I endonuclease and gave two digested fragments at 243- and 63-bp (**Figs. 4 and 5**).

The existence of allelic variants in *FSHR* gene reported in cattle [4], [5], [12], [13] and [14], indicated that the *FSHR* gene is polymorphic. These changes in the molecular structure of the *FSHR* gene cause desensitization of the *FSHR* receptors in the cell membrane which results in less efficient hormone signal transmission [15] and [16].

Marson et al., 2005 [4] genetically characterized a population of European-Zebu composite beef heifers, using RFLP markers of *FSHR* gene. The observed genotypic frequencies for the *FSHR* gene varied from 0.075 to 0.347 (mean of 23%), 0.455 to 0.792 (mean of 58%) and 0.132 to 0.273 (mean of 19%) for genotypes GG, CG and CC, respectively, giving a greater frequency of heterozygosis for all of the breed compositions.

Campagnari, 2002 [17] investigated the polymorphisms of *FSHR* gene by PCR-RFLP in different cattle breed compositions. The polymorphism site analysis from digestion with *Alu*I restriction endonucleases allowed the genotype identification for *FSHR* gene; GG, CG and CC. They reported higher values for genotype GG (0.490). For Nellore breeding, the frequency of heterozygotes reported in their study was lower than those in the results obtained by Marson et al., 2005 [4] and Marson et al., 2008 [5].

To identify a predictor to forecast superovulation response on the basis of associations between superovulation performance and gene polymorphism, variation in the bovine *FSHR* gene was investigated by PCR-SSCP and DNA sequencing [18]. One SNP of G278A located in the 5′ upstream region of bovine *FSHR* gene was found in Chinese Holstein cows. Cows with CC genotype had a significant increase in the total number of ova (P <0.01) and produced more transferable embryos (P <0.01) than those of the CD and DD genotypes.

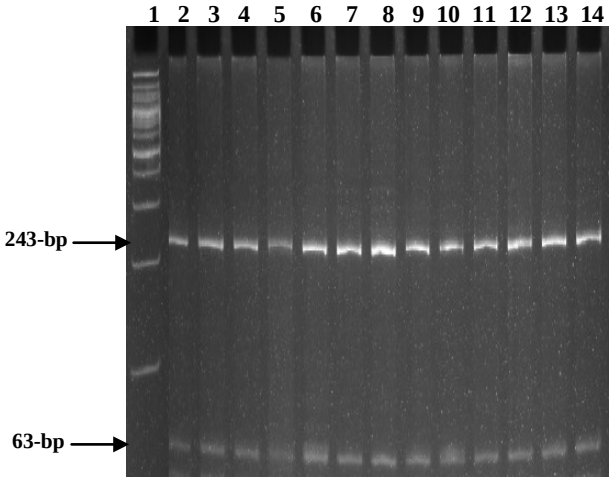

Fig. 4. The electrophoretic pattern obtained after digestion of PCR amplified buffalo *FSHR* with *Alu*I restriction enzyme (on polyacrylamide gel).

Lane 1: 100-bp ladder marker
Lanes 2-14: Homozygous CC genotypes showed two digested fragments at 243- and 63-bp

LHR gene

The primers used in this study flanked a 303-bp fragment from exon 11 of Egyptian buffalo *LHR* gene. The amplified fragments obtained from all tested buffalo DNA (100 animals) were at 303-bp (**Fig. 6**)

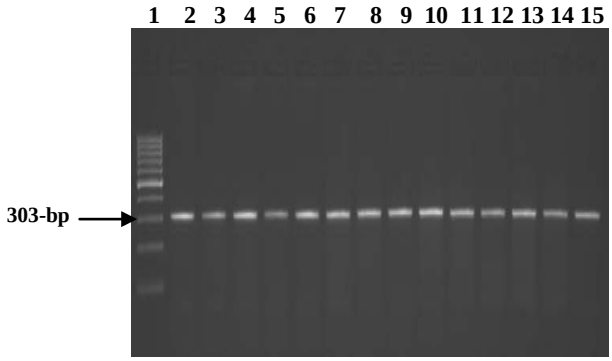

Fig. 6. Ethidium bromide-stained gel of PCR products representing amplification of *LHR* gene in Egyptian buffalo.

Lane 1: 100-bp ladder marker
Lanes 2-15: 303-bp PCR products amplified from Egyptian buffalo DNA

CTGCCTCCCTCAAGGTGCCCCTCATCACTGTGTCCAAGTCAAAGATCCTCCTGGTCCTGTTCTACCCCATCAACT
CCTGTGCCAACCCCTTCCTCTATGCCATCTTCACCAAGAACTTCCGCAGGGATTTCTTCATTCTGCTGAGCAAGT
TTGGCTGCTATGAAGTGCAAGCCCAGACCTATAGGTCAGAAACCTCATCCACTGCCCACAACTTTCATCCAAGG
AATGGTCACTGCCCCCC**AG^CT**CCCAGGGTTACCAGTGGTTCCAATTACACACTTATCCCCCTAAGACATTTAG
CCAAGAACT

Fig. 5. Endonuclease restriction of Egyptian buffalo *F*SHR using FastPCR

In order to verify the PCR product, two-way sequence analysis of the *LHR* amplified PCR products of buffalo DNA was conducted. The buffalo amplicon obtained was found to be 303-bp (**Fig. 7**). The Egyptian buffalo *LHR* nucleotide sequence was submitted to nucleotide sequences database NCBI/ Bankit/GenBank and has the accession number **JQ885687**.

The sequence alignment of Egyptian buffalo *LHR* with published sequence (accession number: DQ858170.1, *Bubalus bubalis*) was carried out using BLAST and showed that the Egyptian buffalo *LHR* possess identities at 98% with five SNPs; (**T/C**) at position 34, (**G/A**) at position 60, (**A/G**) at position 61, (**T/C**) at position 88 and (**A/T**) at position 285 in the reverse-primer region (**Fig. 8**).

These PCR amplified fragments (303-bp) were digested with *HhaI* endonuclease. Depending on the presence or absence of the restriction site at position 155^156 (GCG^C), we can easily differentiate between 3 different genotypes; TT with undigested one fragment at 303-bp, CC with two digested fragments at 155-and 148-bp and TC with three digested fragments at 303-, 155- and 148-bp.

All animals investigated in this study are genotyped as **TT** where all tested buffalo DNA amplified fragments with 303-bp were digested with *Hha*I endonuclease and gave one undigested fragment at 303-bp.

Marson et al., 2005 [4] genetically characterized a population of European-Zebu composite beef heifers, using RFLP markers of *LHR* gene. The observed genotypic frequencies for the *LHR* gene varied from 0 to 0.091, 0.366 to 0.849 and 0.151 to 0.574, respectively, for genotypes TT, CT and CC, in the six different breed types, giving the highest frequency of heterozygosis in tested animals. Higher values for TT (0.540) and lower values for CC (0.030) were reported by Milazzotto, 2001 [19] for a Nellore population.

Marson et al., 2008 [5] recorded a higher frequency of heterozygotes among all European-Zebu composite beef heifers for the *LHR* gene. Lower values of heterozygotes from 0.430 and 0.174 were found by Milazzotto, 2001 [19] and Carvalho et al., 2004 [20], respectively for Nellore females.

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CAAACTGACAGTCCCCCGCTTTCTCATGTGCAATCTCTCCTTTGCAGACTTCTGCATGGGACTCTACCTGCTGCT
CATTGCCTCAGTTGATGCCCAGACCAAAGGCCAGTATTACAACCATGCCATAGACTGGCAGACAGGGAGTGGG
TGCAGCACGGCTGGCTTTTTCACTGTGTTTGCAAGTGAACTCTCTGTCTACACCCTCACAGTCATCACACTAGA
AAGATGGCACACCATCACCTATGCTATTCAACTGGACCAAAAGCTGCGACTGAAACATGCCATTCCAGTCATG
CTCGGAGG
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Fig. 7. The sequence analysis of Egyptian buffalo *LHR* amplified fragment.

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Query  1    CAAACTGACAGTCCCCCGCTTTCTCATGTGCAATCTCTCCTTTGCAGACTTCTGCATGGG  60
            ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  201  CAAACTGACAGTCCCCCGCTTTCTCATGTGCAACCTCTCCTTTGCAGACTTCTGCATGGA  260

Query  61   ACTCTACCTGCTGCTCATTGCCTCAGTTGATGCCCAGACCAAAGGCCAGTATTACAACCA  120
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  261  GCTCTACCTGCTGCTCATTGCCTCAGTCGATGCCCAGACCAAAGGCCAGTATTACAACCA  320

Query  121  TGCCATAGACTGGCAGACAGGGAGTGGGTGCAGCACGGCTGGCTTTTTCACTGTGTTTGC  180
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Query  181  AAGTGAACTCTCTGTCTACACCCTCACAGTCATCACACTAGAAAGATGGCACACCATCAC  240
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Sbjct  381  AAGTGAACTCTCTGTCTACACCCTCACAGTCATCACACTAGAAAGATGGCACACCATCAC  440

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            |||||||||||||||||||||||||||||||||||||||||||  ||||||||||||||||
Sbjct  441  CTATGCTATTCAACTGGACCAAAAGCTGCGACTGAAACATGCCTTTCCAGTCATGCTCGG  500

Query  301  AGG  303
            |||
Sbjct  501  AGG  503
```

Fig. 8. Sequence alignment of Egyptian buffalo *LHR* with published sequence.

The polymorphism of *LHR* and its effects on sperm quality traits including semen volume per ejaculate (VOL), sperm density (SD), fresh sperm motility (FSM), thawed sperm motility (TSM), acrosome integrity rate (AIR) and abnormal sperm rate (ASR) was evaluated by Sun et al., 2012 [21] in Chinese Holstein bulls. Two SNPs; A51703G and G51656T; in intron 9 of LHR were identified. With regards to genotype and its interaction with age, only the SNP of G51656T in *LHR* gene had significant effect on SD ($P < 0.05$, $P < 0.01$; respectively). The association result showed that bulls with AG genotype had higher FSM than bulls with AA and GG genotype in *LHR* at 51703 locus ($P<0.10$), and bulls with GG genotype had higher SD than bulls with TT genotype in *LHR* at G51656T locus ($P<0.10$).

***ERα* gene**

The primers used in this study flanked a 248-bp fragment from the putative promoter for exon C of Egyptian buffalo *ERα* gene. The amplified fragments obtained from all tested buffalo DNA (100 animals) were at 248-bp (**Fig. 9**)

In order to verify the PCR product, two-way sequence analysis of the *ERα* amplified PCR product of buffalo DNA was conducted. The buffalo amplicon obtained was found to be 248-bp (**Fig. 10**). The Egyptian buffalo *LHR* nucleotide sequences of two different alleles G and A were submitted to nucleotide sequences database NCBI/ Bankit/GenBank and have the accession numbers **JQ308795** and **JQ308796**, respectively.

The sequence alignment of Egyptian buffalo *ERα* with published sequence (accession number. AY340597.1, *Bison bonasus*) was carried out using BLAST and showed that G-allele allele possess identities at 99% with 3 SNPs; one SNP (**G/A**) at position 89, and 2 SNPs (**A/G**) at positions 200 and 201 and A-allele possess identities at 98% with these 3 SNPs found in allele G in addition to another SNP (**A/G**) at positions 165 (**Fig. 11**)

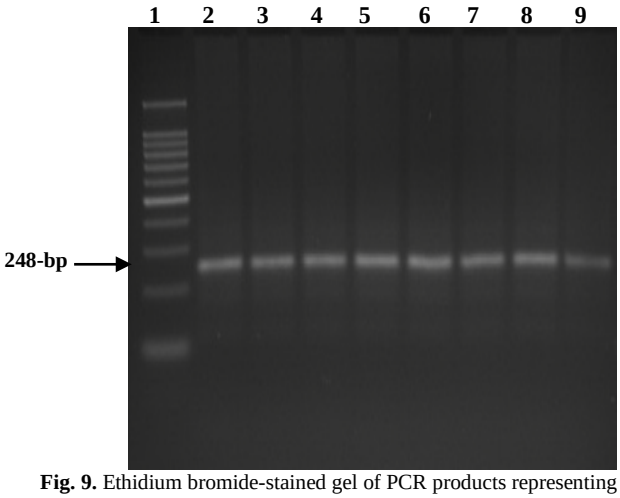

Fig. 9. Ethidium bromide-stained gel of PCR products representing amplification of *ERα* gene in Egyptian buffalo.

Lane 1: 100-bp ladder marker
Lanes 2-9: 248-bp PCR products amplified from Egyptian buffalo DNA

These PCR amplified fragments (248-bp) were digested with *Bgl*I endonuclease. Depending on the presence or absence of the restriction site at position 171^172 (**GCCNNNN^NGGC**), we can easily differentiate between 3 different genotypes; AA with undigested fragment at 248-bp, GG with two digested fragments at 171- and 77-bp and AG with three digested fragments at 248-, 171- and 77-bp

TTTGGTTAACGAGGTGGAGGAATATTACATTTCAGTTGGAAACACATCCCTAGAATGCCAAAACATTTATTCCA
AAGTCTGGTTTCCTGGTGCAATGGGAGGCACGGCAACAGTGTGTCTGTTCAGAGACAGGGGGATGGTGAGGAG
GGACAGGGCGGGCTGGG**(G/A)**CCAGCAAGGCATCTGATCCAAGTGGATCCCAGAAAACTTTTATTGTTAAATT
ATATTCTTCAGGAAAAACCACCTGTGTCACA

Fig. 10. The sequence analysis of two alleles G and A of Egyptian buffalo *ERα* amplified fragment.

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Query  23   TATTACATTTCAGTTGGAAACACATCCCTAGAATGCCAAAACATTTATTCCAAAGTCTGG  82
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TATTACATTTCAGTTGGAAACACATCCCTAGAATGCCAAAACATTTATTCCAAAGTCTGG  60

Query  83   TTTCCTGGTGCAATGGGAGGCACGGCAACAGTGTGTCTGTTCAGAGACAGGGGGATGGTG  142
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TTTCCTAGTGCAATGGGAGGCACGGCAACAGTGTGTCTGTTCAGAGACAGGGGGATGGTG  120

Query  143  AGGAGGGACAGGGCGGGCTGGGACCAGCAAGGCATCTGATCCAAGTGGATCCCAGAAAAC  202
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Sbjct  121  AGGAGGGACAGGGCGGGCTGGGGCCAGCAAGGCATCTGATCCAAGTGGATCCCAGAAGGC  180

Query  203  TTTTATTGTTAAATTATATTCTTCAGGAAAAACCACCTGTGTCACA  248
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TTTTATTGTTAAATTATATTCTTCAGGAAAAACCACCTGTGTCACA  226
```

Fig. 11. Sequence alignment of Egyptian buffalo *ERα* allele A with published sequence.

18 of 100 investigated Egyptian buffaloes in this study (18%) are genotyped as A**G** with three digested fragments at 248-, 171- and 77-bp and the remaining 82 animals (82%) are genotyped as G**G** with two digested fragments at 171- and 77-bp (**Figs. 12** and **13**).

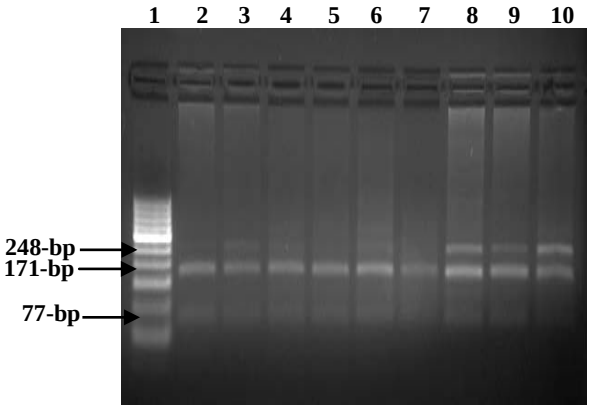

Fig. 12. The electrophoretic pattern obtained after digestion of PCR amplified buffalo *ERα* gene with *Bgl*I restriction enzyme.

Lane 1: 50-bp ladder marker
Lanes 2 and 4-7: Homozygous GG genotype showed two restricted fragments at 171- and 77-bp.
Lanes 3 and 8-10: Heterozygous AG genotype showed three restricted fragments at 248-, 171- and 77-bp

In mammals, estrogens are known to regulate many vital processes, such as reproduction, cell growth, cell differentiation, mammary glands development, lactogenesis and oncogenesis [22]. Estrogen receptors (*ER*) and their genes are considered candidates for the markers of production and functional traits in farm animals. Two isoforms of the estrogen receptor are known (α and β); each of them coded by a separate gene localized on different chromosomes. In humans and animals, *ERα* gene was found in many tissues and the expression sites are in the reproductive organs; the uterus, vagina and ovaries in females and in the testes, epididymis and prostate in males [23].

Szreder and Zwierzchowski, 2004 [9] amplified seven overlapping fragments of the 5' region of the bovine *ERα* gene. These fragments were composed of 2853-bp sequence which was deposited in the GenBank database (accession number: AY340597.1). The sequenced fragment included the noncoding exons A, B, C, their putative promoters and a part of the coding exon 1. A polymorphism within the 5' region of the bovine *ER* gene (A/G transition), which could be recognized with RFLP-*Bgl*I, lying upstream to the exon C, was identified.

CONCLUSION

Animal breeding is based on population genetics as most of the economically important traits have polygenetic nature. Molecular genetics provides valuable information which could contribute to the knowledge of genes underlying quantitative production traits. In view of the lack of studies about the gene polymorphisms of fertility genes in Egyptian buffalo, the objective of the present research was to detect RFLPs in *FSHR*, *LHR* and *ERα* genes. However, the important role of these genes in fertility performance is well known, thus their polymorphisms and interaction with the fertility trait should be the subject of further research.

To the best of our knowledge, this is the first published data on the genetic characterization and nucleotide sequences of fertility genes in Egyptian buffalo. The nucleotide sequences of Egyptian buffalo *FSHR*C, *LHR*T, *ERα*G and *ERα*A alleles were submitted to nucleotide sequences database NCBI/ Bankit/GenBank and have the accession numbers JX049145, JQ885687, JQ308795 and JQ308796, respectively.

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TTTGGTTAACGAGGTGGAGGAATATTACATTTCAGTTGGAAACACATCCCTAGAATGCCAAAACATTTATTCCA
AAGTCTGGTTTCCTGGTGCAATGGGAGGCACGGCAACAGTGTGTCTGTTCAGAGACAGGGGGATGGTGAGGAG
GGACAGGGCGGGCTGGG**GCCAGCA^AGGC**ATCTGATCCAAGTGGATCCCAGAAAACTTTTATTGTTAAATTAT
ATTCTTCAGGAAAAACCACCTGTGTCACA

Fig. 13. Endonuclease restriction of Egyptian buffalo *ERα* allele G using FastPCR

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