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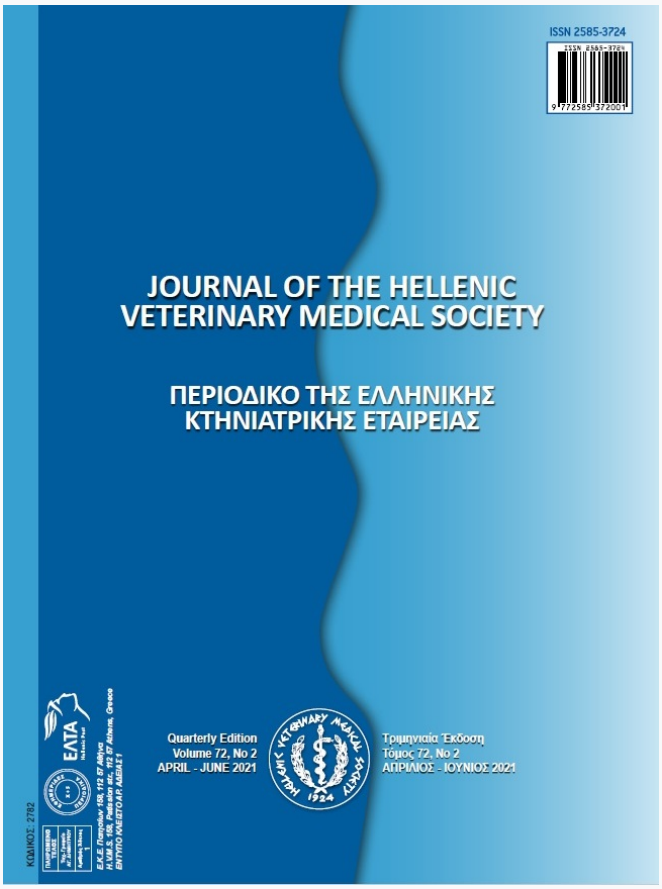

Genetic polymorphisms in FSHR/ALUI and ESRα /BG1I loci and their association with repeat breeder incidence in buffalo

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Research article
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Genetic polymorphisms in *FSHR*/*ALUI* and *ESRα* /*BG1I* loci and their association with repeat breeder incidence in buffalo

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ABSTRACT: The objectives of this study were to explore the association between *FSHR* and *ESR*α genes polymorphism and repeat breeder incidence in Buffalo. DNA was extracted from 243 (96 normal fertile and 147 repeat breeder) Egyptian buffaloes. PCR-*Alu*Iof 306-bp of *FSHR* gene yielded two digested (243 and 63 bp) fragments for the genotype CC, four fragments (243, 193, 63, and 50 bp) for the genotype CG and three fragments (193, 63, and 50 bp) for the genotype GG. Logistic regression analysis presented a significant association of C and G alleles with the incidence of repeat breeder; where the G allele showed a significantly higher incidence compared to C allele in repeat breeder heifers. DNA sequencing of 306 bp of the*FSHR* gene confirmed the polymorphic patterns attained by RFLP analysis; where C/G SNP was detected and changed threonine into serine amino acid. PCR-RFLP/*Bg1I* of 248-bpfromthe*ESR*α gene revealed one monomorphic GG genotype (171, 77 bp) confirmed by DNA sequencing. There were no detected SNPs in all enrolled animals. The results herein suggest the effectiveness of*FSHR*/*Alu*Ilocus polymorphism as a candidate for the incidence of repeat breeder in buffalo than*ESR*α/*Bg1I* one results in marker-assisted selection (MAS) against infertile animals

Keywords: *FSHR, ESRα*genes, repeat breeder, buffaloes, PCR-RFLP, DNA sequencing.

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INTRODUCTION

Water buffaloes were firstly domesticated in India 5000 years ago to provide a source of milk powder. Two groups of buffaloes were identified: swamp buffaloes (2n=48) that were developed chiefly for draught purpose and river buffaloes(2n=50) served as milking animals for dairy industries (Kierstein et al., 2004).They have essential effects on the economy of several countries including livestock and agricultural industries (Yindee et al., 2010).According to the last estimates of FAO (2013), there are about 195 million buffaloes in the world, 97% of them in Asia, 2% in Africa mainly in Egypt, and 0.2% in Europe mainly in Italy.The reproductive performance of any animal is affected by the genotype of both sexes including sire and dam (Mamta & Menaka, 2015).

Repeat breeder syndrome remainsthe most important type of infertility in domestic animals particularly in buffalo as it has a great economic impact for animal breeders. It contributes also to long calving intervals, increasing culling rates, and replacement costs (Garcia-Ispierto et al., 2007). Amiridis et al., (2009)classified the causes of repeat breeders into two major categories: fertilization failure and early embryonic death. The development of DNA markers is an essential tool for applications in animal breeding (Stamoulis et al., 2010;Arslan et al., 2019).Nguyen (2010) cited that buffalo suffer from many reproductive problems such as attaining puberty later than cattle, little numbers of follicles on their ovaries, unnoticed heat, ovulation at various times, seasonal breeding, and long postpartum anestrus. In buffaloes, fertility problems like repeat breeder are not easily recognized (Azawi et al., 2008). This syndrome are responsible for long service period and inter-calving interval that leads to low milk and low calf crop resulting in greater economic losses in the dairy industry. To minimize these losses, early and accurate diagnosis of the causes of these syndromes followed by appropriate timely interventions are required (Singh et al., 2008).

It was established that, chromosomal aberrations and autosomal recessive genes may be accounted for 20% of total early embryonic death that causes the repeat breeder condition (King, 1990). Additionally the Robertsonian translocation (t1/29) causes lower conception rate and early abortion which also caused females to be repeat breeder (Popescu & Pech, 1991). Genetic evaluation of animal reproductive performance depends chiefly on molecular markers technologies that identify genes related to reproductive efficiency (Beuzen et al., 2000). On the other hand, genetic polymorphisms have an important role in many fields of animal breeding (Stoneking, 2001). The development of breeding program strategies requires the description of the genetic structure of populations, breeds and species because it provides information that is necessary for genetic conservation programs. These characterizations offer support and intensify the traditional selection methods (Vasconcellos et al., 2003).

Follicle-stimulating hormone receptor (*FSHR*) gene ispositioned on chromosome number 11 and consists of 10 exons and 11 introns, the first 9 exons encompass the extracellular domain while exon 10 encloses the transmembrane domain (Houde et al., 1994). It is expressed in the ovaries of females and performs its actions by joining with follicle-stimulating hormone (Themmen & Huhtaniemi, 2000), to stimulate the gametogenesis process (Simoni et al., 1997). It has a major role in follicular development in the ovary (George et al., 2011). The FSHR gene is the main determinant of ovarian responsiveness to FSH for the induction of ovulation in females (Yang et al., 2012).

Estrogen receptors (ESR) are composed of two isoforms: ESR1 and ESR2 and each one of them is yielded from a separate gene and is situated on different chromosomes. They are recognized in multiple tissues but uterus, vagina, and ovaries are the chief positions of their expression in females (Enmark and Gustafsson, 1998). Estrogen receptor alpha (*ESRα*) gene is localized on chromosome 9 and contains 8 coding exons (Szreder et al., 2011). It plays a role in the regulation of reproduction, development of the mammary gland (Rani et al., 2016). The *ESR* gene revealed a strong affinity to impact the activity of animals during a period of estrus due to its presence in the ovary (Schams and Berisha, 2002). Estrogen receptors are nuclear receptors (Bjornstrom and Sjoberg, 2005). Previous studies reported the association between *FSHR* and ESR genes polymorphisms and reproductive problems in buffalo; however,controversial resultswerealso obtained(Yang et al., 2010; Othman & Abdel-Samad, 2013; Sosa et al., 2015; Rani et al., 2016; Shafik et al., 2017).

Consequently, the main objectives of this study were to detect polymorphisms of *FSHR* and *ESR*α-genes and their association with the incidence of repeat breeder in Egyptian buffalo heifers using PCR-RFLP and DNA sequencing techniques.

MATERIALS AND METHODS

Animals and Experimental samples

The present study was conducted on a total of 243 (96 normal fertile and 147 repeat breeder) Egyptian buffaloes (*Bubalus bubalis*) aged from 2 to 3 years old. Animals were selected from three localities: A buffalo nucleus herd kept in Nataff-Gedeed Station, Mahalet-Mousa Farm, Agricultural Research Centre, Ganat El-Reida, and El-Noor farms, Ismailia governorate. Based on farm history, animals conceived from one or two successive inseminations and became pregnant were represented as a normal fertile group. While animals that had not conceived after three or-more services and associated with true estrus (heat) every 21-25 days was considered as a repeat breeder. Blood samples were collected from the jugular vein into sterilized vacutainer tubes containing EDTA as an anticoagulant and then stored at −20°C for genomic DNA extraction. Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University approved the protocol of the study..

Genomic DNA extraction and PCR

Genomic DNA was extracted from the leucocytes using the Gene JET Genomic DNA purification kit following the manufacturer protocol (Thermo Scientific,Lithuania). The quality of the extracted DNA was assessed by 1% agarose gel electrophoresis. A 306-bp fragment from exon 10 of the*FSHR* gene and a 248 bp from the putative promoter of *ESRα* gene were amplified by PCR using primers shown in Table 1. PCR was carried out in a volume of 50 µl containing 19 µl H_2O, 1.5 µl forward primer, 1.5 µl reverse primer, 3 µl DNA and 25 µl PCR master mix (Bioline, England). The conditions of PCR program were shown in Table 2.Then PCR products were resolved by electrophoresis stained with ethidium bromide, and visualized using UV light of gel documentation system.

Table 1. Forward and reverse primers sequence for *FSHR* and *ESRα* genes, annealing temperatures and, size of PCR amplicon.

Gene	Primers		Annealing temperature (°C)	Size of PCR product (bp)	Reference
	Forward (5′-3′)	Reverse (5′-3′)			
FSHR (part of exon 10)	5′-CTGCCTCCCTCA AGGTGCCCCTC-3′	5′-AGTTCTTGG CTA AATGTCTTAGGGGG-3′	60	306	Marson et al., (2008)
ESRα (part of putative promoter)	5′-TTTGGTTAACG AGGTGGAG-3′	5′-TGTGACACAG GTGGTTTTTC-3′	56	248	Szreder & Zwierzchowski, (2004)

Table 2. Polymerase chain reaction (PCR) condition for *FSHR* and *ESRα* genes.

Gene	Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
FSHR (part of exon 10)	95/5 min	40	95/30 s	60/30 s	72/30 s	72/8 min
ESRα (part of putative promoter)	95/5 min	40	95/30 s	55/30 s		

PCR-RFLP genotyping

The PCR products were digested with the following restriction enzymes; *AluI* (New England Biolabs Inc) for the*FSHR* gene and *Bg1I* (Thermo Scientific,Lithuania) for *ESRα* gene with incubation at 37 °C for 15 min. The cleaved fragments were detected by 2% agarose gel electrophoresis and visualized under UV using a gel documentation system. The RFLP reaction mixture was carried out in 30 µl consisted of 10 µl PCR product, 1 µl restriction enzyme, 10 µl 10× buffer and 9 µl H_2O (dd water).

DNA sequencing

PCR products of normal and repeat breeder animals with different patterns attained by RFLP analysis were sequenced. The PCR bands with expected size were purified using the PCR purification kit following the manufacturer protocol (Jena Bioscience # pp-201×s/Germany) using the method described by Vogelstein and Gillespie (1979). The purified PCR products were sent to be sequenced in one forward direction using ABI 3730XL DNA automated sequencer (Applied Biosystem, USA). The obtained se-

quences were inspected using Chromas software. Sequence analysis and alignment were carried out using NCBI/BLAST and CLC Main Workbench7 software. Ambiguous sequences at the beginning and extreme end of each sequence were trimmed to avoid possible errors in base calling. The Sequences were analyzed using the Chromas Lite 2.1 program (http://technelysium.com.au/?page_id=13) (Altschul et al., 1990). The identity of the sequenced PCR products was examined using BLAST search against the GenBank database of buffalo (*Bubalus bubalis)* and cattle *(Bos taurus)*,(http://BLAST.ncbi.nlm.nih.gov/BLAST.cgi). The alignments and assembly of the sequences were performed using NCBI.

Data analysis

Gene and genotype frequencies of *FSHR* and *ESRα* genes were calculated according to equations described by Falconer and Macky (1997). Chi-square ($\chi 2$) used to check the Hardy-Weinberg equilibrium. The association between repeat breeder and the identified SNPs was assessed by unconditional multiple logistic regression modelsto obtain the odds ratio (OR) and the corresponding 95% confidence interval (CI). Codominant, dominant, recessive, overdominant and log-additive models were used to avoid the assumption of genetic models. The age and weight of fertile and repeat breeder animals were also adjusted and the statistical analyses were performed using statistical packagefor thesocial sciences (SPSS) software (version 21) **(**SPSS,2004).

RESULTS

The genomic DNA from 243 (96 normal fertile and 147 repeat breeder) buffaloes was extracted to amplify 306 bp (part of exon 10) of *FSHR* gene. The restriction digestion analysis of PCR products conducted with *AluI* endonuclease revealed three genotypes: the CC genotype with two digested fragments: 243 and 63bp, four digested fragments: 243, 193, 63 and 50 bp for the genotype CG and the GG genotype with three digested fragments: 193, 63 and 50 bp (Figure 1).The genotypic and allelic frequencies of the*FSHR* gene were calculated and presented in Table 3. For 96 normal fertile buffaloes, the genotypic frequencies of CC, CG, and GG genotypes were 34.4%, 39.6%, and 26% respectively. While in 147 repeat breeder heifers the genotypic frequencies were 21.1%, 36.7% and 42.2% respectively. Allelic frequencies of C and G alleles were 54% and 46% in normal animals and 39% and 61% in repeat breeders. The $\chi 2$-test presented the obtained *FSHR* gene genotypic distribution among normal fertile buffaloes that was deviated from Hardy-Weinberg equilibrium ($p<0.05$), while in repeat breeder heifers the genotypic distribution followed Hardy-Weinberg equilibrium ($p>0.05$). The different band patterns obtained by the RFLP marker were DNA sequenced. Nucleotide sequences for the forward primer direction of *FSHR* gene (306 bp) alignment between normal fertile and repeat breeder buffalo heifers were carried out using CLC Main Workbench7 program. The results confirmed the digested polymorphic patterns attained by RFLP analysis; where it was revealed the presence of C/G non-synonymous transversion SNP replaced threonine into serine (Figure 3).

As revealed in Table 4, logistic regression analysis presented a significant association of C and G alleles of the *FSHR* gene with incidence of repeat breeder (P=0.002). In repeat breeder heifers, the G allele showed a significantly higher incidence compared to C allele: 61% versus 39%. Indicating that heifers carrying the risk allele G have a higher susceptibility to repeat breeder in comparison with C allele carriers and increased the OR value of the risk for infertility to 1.81 with 95% CI= 1.26-2.62. Meanwhile, in normal fertile heifers, G allele appeared with lower frequency when compared to C allele: 46% versus 54%. The C>G transversion detected SNP showed significant association when tested under different genetic models. With the codominant model (P=0.016), animals with a homozygous GG genotype at this locus had an OR of 1.00 with 95% CI for being repeat breeder compared to CG genotype that had OR = 0.57 with 95% CI= 0.31-1.07, while CC genotype had OR of 0.38 and 95% CI= 0.19-0.74. Under the dominant model of the C>G SNP (G/G versus C/G+C/C), it showed a highly significant association (P=0.0095) with repeat breeder with OR of 0.48 and 95% CI= 0.28-0.85. Meanwhile, the recessive model of the*FSHR* gene (G/G+C/G versus C/C) SNP showed a significant association (P=0.022) with repeat breeder with OR of 0.51, 95% CI: 0.29-0.91. The overdominant model (G/G+C/C versus C/G) revealed a non-significant association (P=0.65) with repeat breeder with OR of 0.89 and 95% CI= 0.52-1.50. However, logistic regression models revealed significant associations ($P<0.05$) between codominance, dominance, recessive, over-dominant as well as log-additive effects of C>G SNP and the occurrence of repeat breeder in buffaloes. For this SNP, the log-additive effect (P=0.0041) can be interpreted as every additional copy of the risk

allele G at this locus resulted in an increased risk of repeat breeder by 0.61, 95% CI= 0.44-0.86 in buffalo heifers. Consequently, buffaloes carrying the heterozygous CG genotype are 0.61 more likely to develop repeat breeders compared to reference CC genotype. Furthermore, the mutant G allele is a highly risk allele that increased the susceptibility to repeat breeder by 0.61, CI= 0.44-0.86 than C allele which is a low risk allele.

Concerning the*ESRα* gene, the genomic DNA was extracted to amplify 248bp (a part of the putative promoter). Restriction analysis of 248 bp PCR products was digested with *Bg1I*. The results showed monomorphic GG pattern with two digested fragments at 171 and 77 bp in all the studied animals (Figure 2). The monomorphic band pattern was confirmed by DNA sequencing; where nucleotide sequence alignment for the forward primer direction of *ESRα* gene (248bp) from normal fertile and repeat breeder buffaloes revealed no variation exists between the sequences of the studied animals (Figure 4).

Table 3. Genotypic and allelic frequencies of the *FSHR* gene in normal fertile and repeat breeder animals.

Animals	No. of animals	Number/frequency of genotypes%			Allele frequency %		Risk allele	χ2 (HWE)	P-value
		CC	CG	GG	C	G			
Fertile	96	33/34.4	38/39.6	25/26	54	46	G	3.948	0.04692
Repeat breeder	147	31/21.1	54/36.7	62/42.2	39	61		7.852	0.04259

HWE-Hardy Weinberg Equilibrium. Hardy Weinberg test was done using the Pearson's goodness of fit test. P value<0.05 was considered to show significant deviation of the observed genotypes from Hardy-Weinberg proportions.

Table 4. Genotypic and allelic association of C>G SNP of *FSHR* gene polymorphism with repeat breeder incidence under different genetic models.

Comparative models	Genotypes	Fertile (n=96)	Infertile (n=147)	OR (95% CI)	P-value
Codominant	G/G	25 (26%)	62 (42.2%)	1.00 (reference)	0.016
	C/G	38 (39.6%)	54 (36.7%)	0.57 (0.31-1.07)	
	C/C	33 (34.4%)	31 (21.1%)	0.38 (0.19-0.74)	
Dominant	G/G	25 (26%)	62 (42.2%)	1.00 (reference)	0.0095
	C/G-C/C	71 (74%)	85 (57.8%)	0.48 (0.28-0.91)	
Recessive	G/G-C/G	63 (65.6%)	116 (78.9%)	1.00 (reference)	0.022
	C/C	33 (34.4%)	31 (21.1%)	0.51 (0.29-0.91)	
Overdominant	G/G-C/C	58 (60.4%)	93 (36.3%)	1.00 (reference)	0.65
	C/G	38 (39.6%)	54 (63.7%)	0.89 (0.52-1.50)	
Log-additive	---	---	---	0.61 (0.44-0.86)	0.0041
	Allele	Fertile (n=96)	Infertile (n=147)	OR (95% CI)	P-value
FSHR C>G	C	No. 104 % 54%	No. 116 % 39%	1.00 (reference)	0.002
	G	88 46%	178 61%	1.81 (1.26-2.62)	

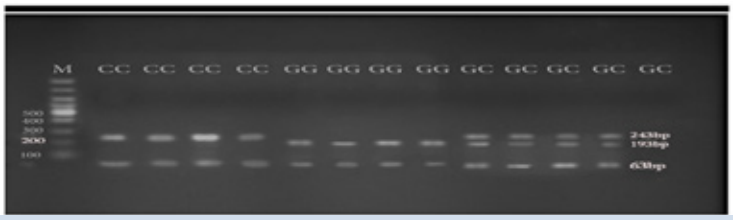

Fig 1. Ethidium bromide stained 2% agarose gel electrophoresis of representative samples of RFLP banding pattern of *FSHR* gene (306-bp) from normal fertile and repeat breeder buffaloes after digestion with *AluI*. M: 100 bp ladder

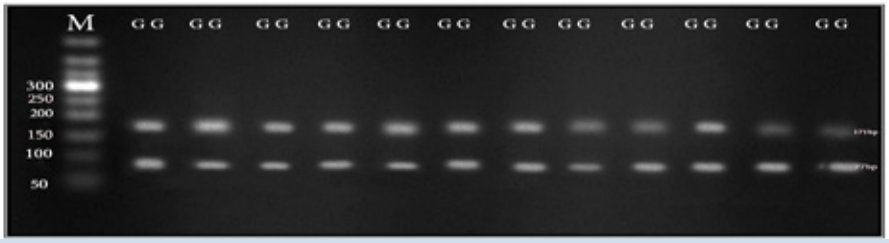

Fig 2. Ethidium bromide stained 2% agarose gel electrophoresis of representative samples of RFLP banding pattern of *ESRα* gene (248 bp) from normal and repeat breeder buffaloes after digestion with *Bg1I*. M: 50 bp ladder

Fig 3. (a) DNA sequence alignment of *FSHR* gene (306 bp) between normal fertile and repeat breeder buffalo heifers using CLC Main Workbench7 program, (b) Alignment of amino acids of *FSHR* gene (part of exon 10-306 bp) between normal fertile and repeat breeder buffalo heifers using BLAST, (c) DNA sequence alignment of ESRα gene (248 bp) between normal fertile and repeat breeder buffaloes using CLC Main Workbench7 program.

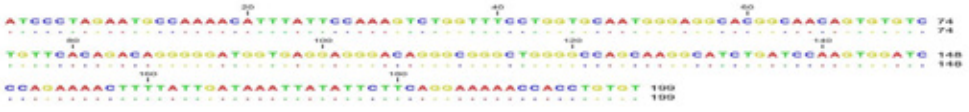

Fig 4. DNA sequence alignment of *ESRα* gene between normal fertile and repeat breeder buffaloes using CLC Main Workbench7 program.

DISCUSSION

In a breeding system, reproductive efficiency has attained remarkable interest, especially in seasonal breeder animals. Buffalo are able to breed throughout the year; however in developing countries, like Egypt, the chance of pregnancy attained by the seasonal trend of ovarian activity is time-limited (Barile, 2005). Buffalo have smaller ovaries, fewer primordial follicles, less over estrous signs than cattle(El-Wishy, 2007; Perera, 2011). This may be attributed to the peak concentrations of progesterone as well as oestradiol-17â is less detected. Field surveys on reproductive disorders revealed that anestrum and repeat breeder were the most common cause of infertility in buffaloes particularly in Egypt (Singh & Sahni, 1995; Ahmed et al., 2012).

In this study, the RFLP analysis revealed the presence of C/G non-synonymous SNP transversion which causes to replace threonine into serine. Interestingly, the logistic regression analysis presented a significant association of C and G alleles of the *FSHR* gene with the incidence of the repeat breeder; where G allele showed a significantly higher incidence compared to C allele. Previous studies reported the association between *FSHR/AluI* locus polymorphisms and the incidence of infertility traits in both cattle and buffaloes populations; however controversial results were also obtained. Moreover, different distribution and frequencies for the attained genotypes; that is may be attributed to the genetic background differences between the studied animals. Regarding the polymorphism in buffaloes, Othman & Abdel-Samad (2013) determined genetic polymorphism of the *FSHR-AluI* gene among healthy buffaloes. They obtained only one CC genotype (243 and 63 bp). According to the results, sequence alignment of the *FSHR* gene showed that the *FSHR* gene in the Egyptian buffaloes possessed identities at 99% with only G/A SNP at position 59 of this gene. Also, Sosa et al., (2015) worked to indicate the effect of *FSHR* gene polymorphism on anestrus and repeat breeder, no significant differences between animals were detected. All animals studied in this study were genotyped as CC. Shafik et al., (2017) determined the association between polymorphisms of the *FSHR* gene and infertility using DNA sequencing and they did not found any SNP in 306 bp in all examined buffaloes.

A number of related studies were carried out in different cattle populations. For instance, Marson et al., (2008) studied the effect of *FSHR* gene polymorphism on sexual brightness among 370 cattle beef heifers. PCR-RFLP/*AluI* yielded three genotypes: GG with three fragments (193, 63, and 50 bp), CG with four fragments (243, 193, 63, and 50 bp) and CC with two fragments (243 and 63 bp). It was found that the heifers with CG genotype presented a 66% pregnancy rate, Meanwhile, GG genotype exhibited 58% and CC genotypes revealed 64%. However, the effect of the *FSHR* gene polymorphism on pregnancy rates between the diverse breeds was not established.Hernandez et al., (2009) tried also to find a variation of the *FSHR* gene in different cross breeds of cattle. The authors used the same primer and restriction enzyme and found different gene and genotypic frequencies in the studied cattle population. They added that, Hardy-Weinberg equilibrium was detected in the Bos indicus and Bos taurus x Bos indicus cattle but not in the Bos taurus group. Moreover, the association between *FSHR* gene polymorphism and superovulation in Chinese Holstein cows was investigated by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing (Yang et al., 2010). The authors reported the presence of G278A and A320T SNPs. They showed also that *FSHR* gene is a possible predictor for superovulation in Chinese Holstein cows.In the same line, Sang et al., (2011) investigated the association between *FSHR* gene polymorphism and sperm quality traits in mature Holstein bulls. The authors added that bulls with AA genotype might have significantly higher sperm quality traits than AT genotype.Andreas et al., (2014) declared also a significant effect at ovulation rate, while individual animals with the CC genotype of *FSHR-AluI* had a higher ovulation rate than CG and GG genotypes.In the same respect, Arslan et al., (2017) reported that a digested fragment of 306 bp of the *FSHR* gene with *AluI* obtained three genotypes with a significant deviation from HWE between low insemination and high insemination cows. On divergence to our results, Omer et al., (2016) characterized Sudanese cattle and obtained only one CC genotype.

Mutations in the promoter region greatly affect the post-transcriptional regulation of gene expression and impact the accuracy of the translation process (Fürbass et al., 1997) Although some mutations are positioned in promoter regions of the genes and they would not change the amino acid sequence of protein products but they play a role in the functions of estrogen as they are found in the transcription factors' binding sites and so leading to an alternation of the expression levels of the genes. According to our re-

sults, there was no association between the*ESRα* gene polymorphism and the incidence of repeat breeder. For the *ESRα* gene, the results herein showed monomorphic GG pattern with two digested fragments at 171, and 77 bp in all the studied animals confirmed by DNA sequencing. Previous studies reported opposing results for the latter polymorphism. For instance Othman & Abdel-Samad (2013)used the same primer, endonuclease enzyme, and amplified the same fragment of *ESR* gene.However,PCR- *Bg1I* 248 bp of the *ESRα* gene elicited two genotypes; GG genotype with two digested fragments: 171 and 77 bp and three fragments 248, 171, and 77 bp for the AG genotype. Additionally,they found that 18% of animals are the AGgenotype and 82% are the GG genotype.On the contrary, the results ofRani et al., (2016) agreed with the obtained results. They used two endonucleases and obtained a monomorphic pattern in all the studied animals.

Exploring the polymorphism was carried out on different regions ofthe *ESR* gene and its association with reproductive traits.Zahmatkesh et al., (2011) digested 245 bp of *ESRα* gene with *BglI* endonuclease, they found three genotypes: AA (245 bp), AG with three digested fragments (168, 77, and 243 bp) and GG had two fragments (168 and, 77 bp) with genotypic frequencies: 0.010, 0.129, and 0.861. Allelic frequencies of A and G were 0.0742 and 0.9257. It was presented that, A/G transition had no significant effect on reproduction traits. In a cohort of cattle population, Szreder &Zwierzchowski (2004)used PCR-RFLP and DNA sequencing to determine the genetic polymorphism of *ESRα* gene among different breeds and obtained AG and GG genotypes, the results of DNA sequencing revealed A/G transition in the promoter region leading to silent mutation. Sangdehi et al., (2015)used also *SnaBI* endonuclease to digest a 340 bp fragment from the promoter region of *ESRα* gene and obtained three genotypes: AA, AG, and GG between four breeds of cattle: Mazandarani, Taleshi, Sistani, and Simmental. The genotypic frequencies were: 0.65, 0.75, 0.35, and 0.91 respectively for AA genotype, 0.30, 0.25, 0.40, and 0.09 for AG genotype and 0.05, 0.0, 0.25, and 0.0 for GG genotype. Kathiravan et al.,2017detected genetic polymorphism of *ESRα* gene among different Indian murrah buffalo breeds. Genomic DNA was extracted to amplify 870 bp from exon 13 that was digested by *MboI* restriction enzyme. All animals enrolled in this study were of monomorphic pattern and genotyped as AA genotype with 614 and 256 bp fragments. This result shows the conservation of *ESRα*/AA genotype in murrah buffaloes.

The limitations of this study should be acknowledged. First, a small sample size may not allow obtaining a concrete conclusion for elucidating the *ESRα* gene polymorphisms. Second, a limited number of candidate gene markers for the incidence of repeat breeder may also influence the conclusion. Third, other buffalo breeds should also be considered. Accordingly, such shortcoming should be considered in further investigations.

In conclusion, a remarkable significant association was detected between *FSHR*/*AluI* locus polymorphism and incidence of repeat breeder in Egyptian buffaloes. The association of observable phenotypic variationin the fertile and repeat breeder buffaloes with identified polymorphisms can potentially be explained by allele-specific differencesin *FSHR* gene expression. These findings suggest that *FSHR/AluI* gene could be used as a marker for early culling of repeat breeder heifers resulting in preventing economic losses afforded by the breeder resulting in efficient marker-assisted selection (MAS).

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CON FLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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