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Theriogenology

Genetic Polymorphism of *cyp19* and *fshr* Genes and Their Association with Ovarian Inactivity and Silent Heat in Egyptian Buffaloes (*Bubalus bubalis*)

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ABSTRACT

The genetic polymorphism of *cyp19* and *fshr* genes might influence fertility performance in cattle and buffalo. This study used PCR-RFLP to pinpoint genetic variations in the *cyp19*and *fshr* genes and their relationship to the ovarian inactivity and silent heat in Egyptian buffaloes. Whole blood samples were taken in vacuum tubes that contain EDTA from female Egyptian buffaloes. Rectal findings and ultrasonography were carried out for 90 buffaloes and were divided into three equal groups, the 1st group is smooth inactive ovaries, 2nd group is silent heat and the 3rd group is a control group. To analyze the genetic variations in the *cyp19* and *fshr* genes, genomic DNA was isolated from blood. Amplification of a snippet of the cyp19 promoter and 230 bp of fshr exon 10 genes which were digested with PvuII and Hin1II (NlaIII) restriction enzymes respectively. Electrophoresis was used to examine the PCR-RFLP pattern, which revealed that all animals displayed the GG genotype as of 187 and 232 bp fragments except two of silent heat animals showed undigested monomorphic pattern at 419 bp which genotyped as AA. The PCR-RFLP pattern for fshr gene revealed that all the investigated buffaloes were polymorphic at 150, 200 bp and genotyped as GA except one silent heat animal has a monomorphic pattern at 230 bp with AA genotype. In conclusion, the current study revealed that there was no association between genetic polymorphism of *cyp19* and *fshr* genes and reproductive disorders of Egyptian buffaloes especially ovarian inactivity and silent heat in Egyptian buffaloes.

Keywords: Anestrum, Buffalo, *cyp19*, *fshr* and PCR-RFLP.

INTRODUCTION

Buffaloes are capable of breeding throughout the year, Nonetheless, the seasonal ovarian activity in several countries including Egypt makes the opportunity of buffaloes to get pregnant is time limited (Kandiel et al., 2013). Buffalo's estrus symptoms are less noticeable than those of cattle whereas the peak concentrations of progesterone and estradiol are less (Singh and Sahni, 1995). Field surveillance on reproductive disorders revealed that anestrus (in forms of prolonged calving interval and silent heat) was the primary factor in buffaloes'

infertility particularly in Egypt (Fooda et al., 2011 and Ahmed et al., 2011).

Among the main obstacles to buffalo reproduction, silent heat is expressed by the main reason why buffalo reproduction is less exploited (Cochran et al., 2013). Silent heat is a state that buffaloes are not display the behavioral signs of estrus, despite the fact that estrus physiological signs are present (Lopez et al., 2004). As a result of silent heat, animals become repeat breeder and lose their estrus regularity and cyclicity, which has a negative impact on their ability to reproduce (Cho et al., 2012). Lower concentration of ovarian estrogens, which are essential for sexual activity, bone density, reproductive growth, and fertility, are one of the physiological agents of true anestrum (Hafez and Hafez, 2000).

The reproductive performance is genetically determined by molecular technology to discover genes and analyzethe variants of these marker genes, the products of which are essential enzymes in metabolic processes that are crucial for physiological pathways and are linked to phenotypes (Beuzen et al., 2000). Cytochrome P450 aromatase, a protein derived from the *cyp19* gene, is the primary enzyme included in the biosynthesis of estrogen. Many vertebrates possess the hemi-containing enzyme cytochrome P450, which is mostly expressed in male and female gonads and various organs such as placenta and brain (Blakemore and Naftolin, 2016). Aromatase converts androgens into estrogens, which is a crucial function for the reproduction physiology (Jędrzejczak et al., 2011). Cattle's long arm of chromosome 10 has been identified as band 2.6, where the bovine *cyp19* gene has been located (Goldammer et al., 1994), in buffaloes, chromosome 11 (Iannuzzi et al., 2001), they consist of 10 exons that vary in size amongst various species from 56 kb to 120 kb. The translation start site is in exon II, and the coding region contains exon II-X (Simpson and Davis, 2001).Mutations in the *cyp19* gene result in an excess of testosterone and a deficiency in estrogen, which may result in inactive ovaries and inadequate follicular growth (Kumar et al., 2009).The *cyp19* gene's genetic variation may affect the fertility capacity of cattle (Fürbass et al., 1997), Indian Murrah buffaloes (Kumar et al., 2009) and Egyptian buffaloes (Abbas et al., 2014).

Endocrinological pathway is a major regulator to the reproductive physiology through hypothalamic– pituitary–gonad axis and its interactions with a consideration that the FSH importance in the maintenance of ovarian function (Sosa et al., 2015). Among the hormones generated by the hypothalamus pituitary gonadal axis, FSH plays a crucial part in oogenesis (Gaviria et al., 2106). FSH promotes aromatase pathway on granulosa cells, which results in the generation of 17-estradiol, and follicle recruitment and growth (Assidi et al., 2013).In order to regulate its own synthesis, there is also increase in inhibin production (Medan et al., 2007). The FSH which is a glycoprotein made up of two subunits. The alpha subunit which is shared by a group of glycoproteins that are closely related, but the beta subunit is unique to each member of the family, giving these hormones a higher level of biological pathogenicity. However, the alpha and beta subunits contribute to the receptor (George et al., 2011). *fshr* gene is displayed in male and female gonads and the effects of FSH are mediated to testicular and ovarian somatic cells via those receptors (Themmen and Hutaniemi, 2000). There are 10 exons and 9 introns in the *fshr* gene (Simoni et al., 1997). FSH controls ovarian follicle growth, differentiation, maturation, and ovulation via attaching to its particular receptor (*fshr*) on the ovarian surface (Yang et al., 2012). Regarding to FSH effect in the maintenance of ovarian function, many types of animals have had their *fshr* gene investigate, including cattle (Houde et al., 1994), sheep (Yarney et al., 1993), horse

(Robert et al., 1994) and donkey (Richard et al., 1997). There is lack of literatures concerning the genetic polymorphism linked to reproductive efficiency in buffaloes (Ramadan et al., 2020), consequently, the purpose of this study was to assess genetic polymorphism, RFLPs effects in the *cyp19*, *fshr* genes regarding ovarian inactivity and silent heat in Egyptian buffaloes.

MATERIALS AND METHODS

This study was assessed and agreed by the Animal Care and Welfare Committee Ethics, University of Sadat City, Egypt (Ethical approval number: VUSC-022-01-21).

Animals

A total of 90 Egyptian buffaloes were used in the current investigation (1st to 4th lactation season ~3-8 years old) at Menoufia governorate, Egypt.Under the same managementalconditions, these animals were raised on small-scale farms. The case history of all buffaloes was takenbefore trans-rectal ultrasonographicexamination for identifying the condition of their ovaries by using a B-mode gray scale scanner (E1V SonoScape, ®China) with an endorectal probe of 8.6 MHz (Kandiel et al., 2013 and Othman et al., 2014). According to ovarian status after gynecological examination, animals were divided into three groups (30 animals) for each group: The 1st group was suffering from ovarian inactivity, the 2nd group was suffering from silent heat andthe 3rd group was the control animals without any gynecological problems. All animals were apparently normal, vaccinated, dewormed and regularly fed a balanced ration.

Blood Sampling and DNA extraction

EDTA anticoagulant vacutainer tubes were used for collection ofblood samples from all animals. Genomic DNA Mini Kit wasused to extract genomic DNA from the blood samples (Blood/Cultured Cell, Geneaid Biotech Ltd., ®Taiwan) based on instructions provided by the manufacturer.

PCR Reaction and DNA Amplification

A DNA fragment that is a part of the cyp19 distal promoter P1.1 was amplified according to Yazdani et al. (2010) using following primers:

Forward,
5' 3'
CAAGGGCCTCATATGGTTCA
Reverse,
5' 3'
CCAGATCAGAACCACCTTTGT

The Exon 10 of *fshr* locus (with size 230 bp) was amplified by PCR using specific forward and reverse primers which described by Othman and Abdel-Samad (2013) as following:

Forward
5' 3'
ATCACGCTGGAAAGATGGCATACC
Reverse
5' 3'
GACATTGAGCACAAGGAGGGAC

The reaction volume (12.5 µl) was used for the PCR, which contained (6.25 µl PCR master mix (Thermo Fisher Scientific Inc. USA), 1 µl forward primer, 1 µl reverse primer, 2.25 µl distilled water (DW) and 2 µl DNA sample). Amplification procedure included an initial start separation cycle at 94°C for 2 min, 35 cycles with steps for denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, polymerization at 72°C for 45 sec, and a final extension cycle at 72°C for 10 min. The PCR results were examined using electrophoresis on a 3% agarose gel with a 0.5X Tris Acetate EDTA Buffer (TAE), which was stained with ethidium bromide (EtBr), and viewed using an E-Gel Imager (SKU: 10157, Life Technologies, The Lab World Group, United States).

Restriction Fragment Length Polymorphism (RFLP) Technique

For genotyping, PCR product from amplified DNA of both *cyp19* and *fshr* genes were digested with PvuIIandHin1 II (NlaIII) restriction enzymes, respectively (New England Biolabs, Inc.).In a total volume of 15 µl (10 µl reaction solution,

1.5 µl enzyme buffers, 0.5 µl enzymes, and 3 µl DW), gene fragments were exposed to restriction enzyme digestion for 15 minutes at 37°C (Dry Bath Incubator-MK200-2, Hangzhou Allsheng Instruments Co., Ltd., Zhejiang). Following digestion, the samples were measured using an E-Gel Imager to see the amplified fragments, as described in the PCR with 3% agarose concentration.

RESULTS

The DNA of Egyptian river buffaloes was extracted from blood samples and a fragment from the *cyp19* distal promoter P1.1 was amplified. This resulted in a 419 bp PCR product that was then digested with the PvuII restriction enzyme. The pattern of PCR-RFLP was then examined by electrophoresis for the three groups. The PCR- RFLP pattern showed that all the animals of first group (ovarian inactivity) (Fig. 1) and animals of third group (control group) (Fig. 2) depicted the *GG* genotype by 187 and 232 bp fragments.

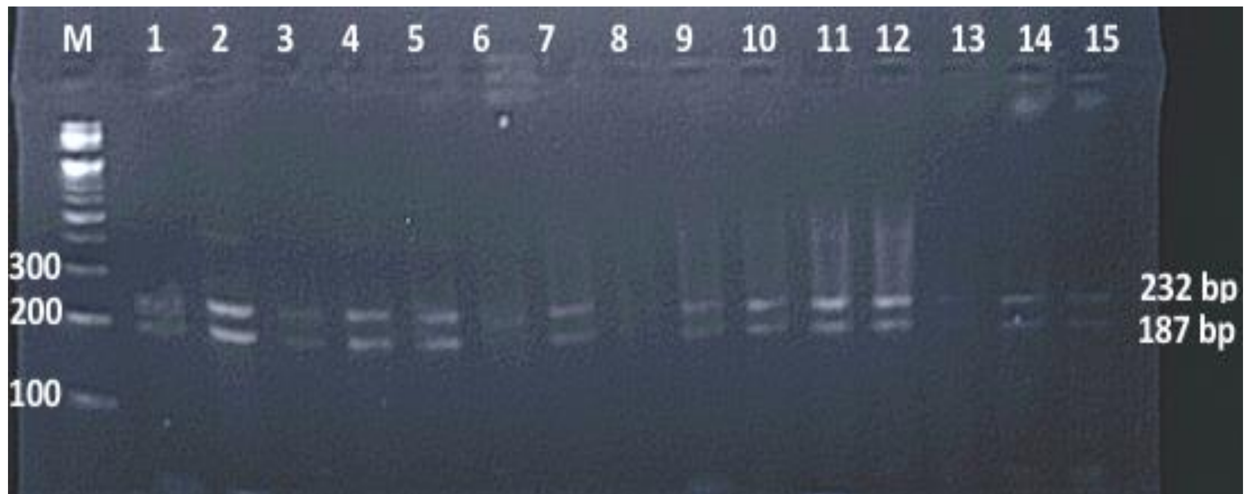

Fig (1): 3% agarose gelDNA electrophoresis pattern was obtained after digestion of PCR amplified *cyp19*gene with PvuII restriction enzyme (lane 1-15), M 100 bp ladder marker in the 1st group.

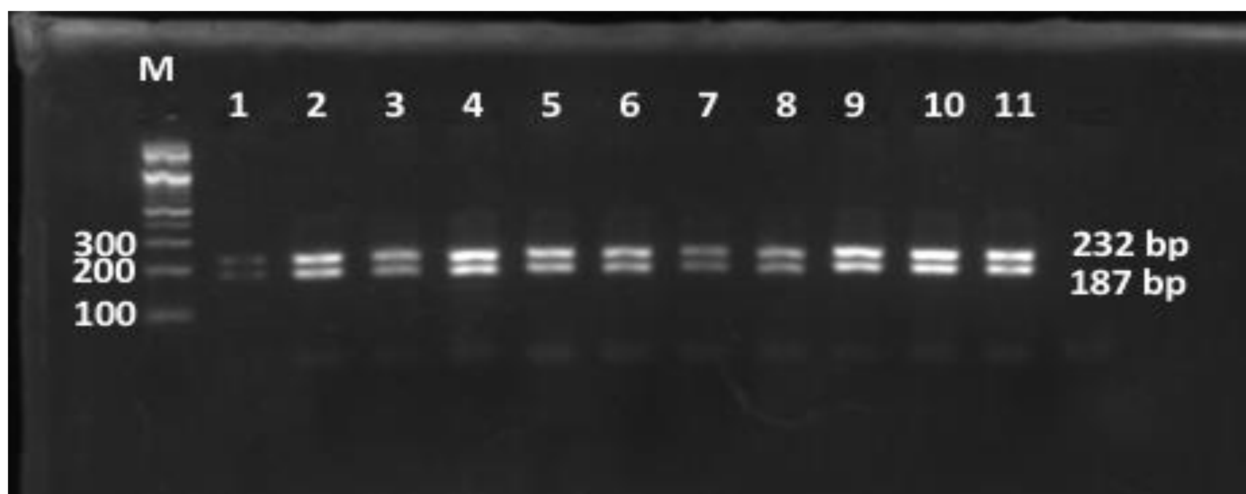

Fig (2): 3% agarose gel DNA electrophoresis pattern was obtained after digestion of PCR amplified *cyp19* gene with PvuII restriction enzyme (lane 1-11), M 100 bp ladder marker in the 3rd group

The second group (silent heat animals) showed only two animals with undigested PCR product a 419 bp with *AA* genotype (Fig. 3) and PCR- RFLP design for other animals revealed that *A* allele was absent in both the homozygous and heterozygous genotypesand represented the *GG* genotype as 187 and 232 bp fragments.

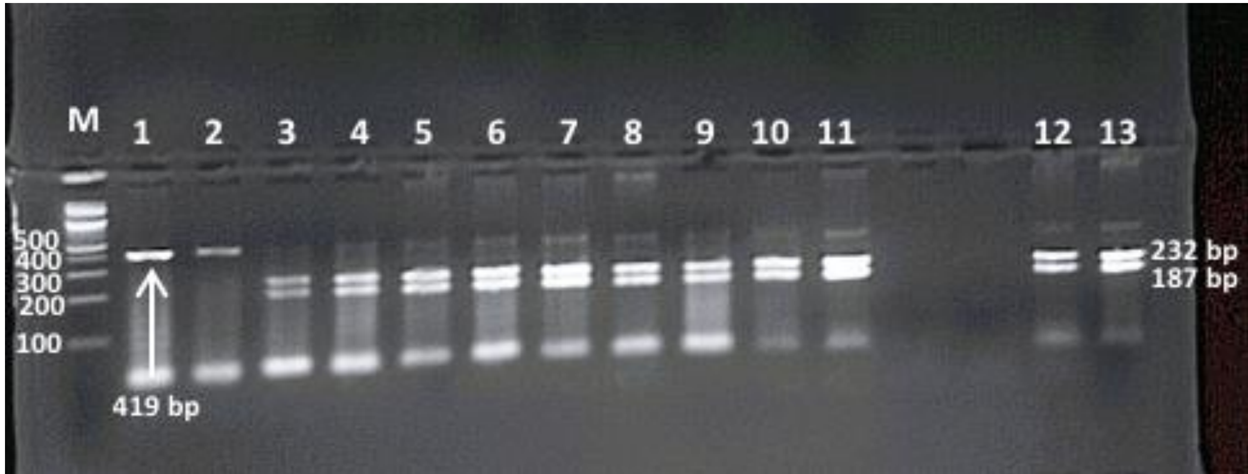

Fig (3): 3% agarose gel DNA electrophoresis pattern was obtained after digestion of PCR amplified *cyp19* gene with PvuII restriction enzyme (lane 1-13) M 100 bp ladder marker in the 2nd group

DNA segments of 230 bp from the Egyptian buffalo's *fshr* exon 10 gene for the three groups (ovarian inactivity, silent heat and control) were amplified. PCR-RFLP was used to identify the polymorphic changes which indicated that all animals showed polymorphic pattern at 150, 200 bp with *GA* genotype except, one animal from silent heat group showed monomorphic pattern at 230 bp which genotyped as *AA* as shown in (Fig. 4, Fig. 5).

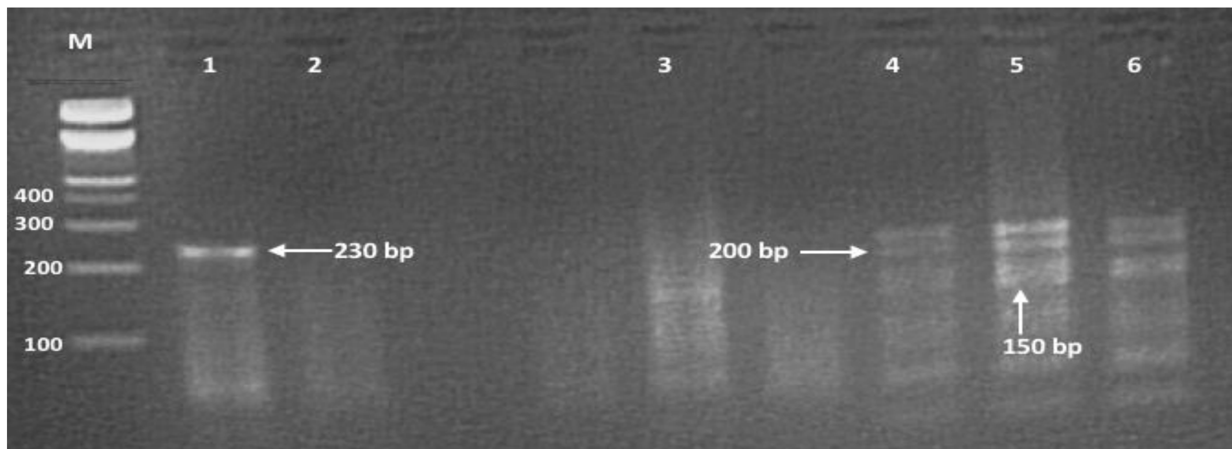

Fig (4): 3% agarose gel DNA electrophoresis pattern was obtained after digestion of PCR amplified *fshr* gene with Hin1II (NlaIII) restriction enzyme (lane 1-6), M 100 bp ladder marker in the 2nd group**.**

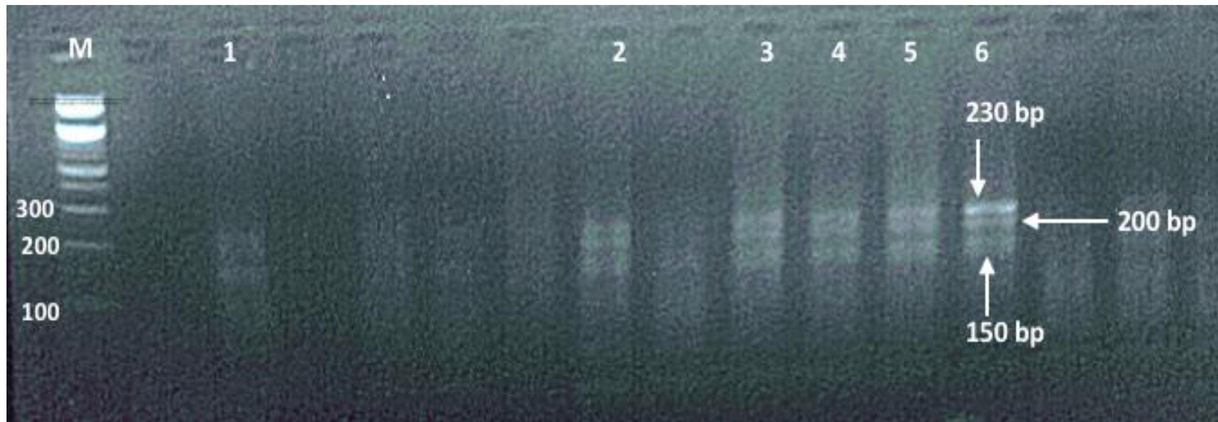

Fig (5): 3% agarose gelDNA electrophoresis pattern was obtained after digestion of PCR amplified *fshr* gene with Hin1II (NlaIII) restriction enzyme (lane 1-6), M 100 bp ladder marker in the 1st group

DISCUSSION

Molecular technology is used to discover genes that are important for reproductive efficiency and to analyze the variations of these marker genes in order to genetically evaluate reproductive performance of animals (Beuzen et al., 2000).Many methods, such as single strand conformation polymorphism (SSCP), might be used to detect single nucleotide polymorphisms (SNPs) (Orita et al., 1989), RFLP (Youil et al., 1995) and denaturing gradient gel electrophoresis (Sheffield et al., 1993). Among these techniques, RFLP is very commonly used and cheaper method (Lan et al., 2006 and Sosa et al., 2015).

In this study the PCR-RFLP pattern showed that 187 and 232 bp fragments from the *GG* genotype were detected in all animals except, two animals from silent heat group showed *AA* homozygous genotype at 419bp. The PCR products provided that the guanidine nucleotide situated at the PvuII restriction site, where the SNP is the target position. In contrast to these findings, Polish Holstein-Friesian cows' *A* allele frequency varied from 92.27 to 93.2% (Szatkowska et al., 2011 and Wierzbicki et al., 2014).

The study revealed that the frequency of *G* allele was greater than the *A* allele. Uttam et al. (2022) indicated that all screened animals had a higher prevalence of the genotype *AA* (58.5%) and allele *A* (0.77) than the genotypes *AB* (37.0%) and *BB* (4.5%). Moreover, Rajab and Abed (2021) recorded that the wild *G* allele's and mutant counterpart *A* allele had a respective frequency 0.51 and 0.49. Furthermore, Aboelenin et al. (2017) demonstratedthat all tested samples had the pure wild composition. In Holstein cattle (Jedrzejczak et al., 2011 and Wierzbicki et al., 2014)revealed the preference of the *A* allele which was greater than 0.92 and this may be due to differences between species despite the great closeness between cows and buffaloes. The study revealed that the absence of *TT* genotype in all animals but on the other hand, Othmanet al. (2014) declared that the genetic pattern *TT* is present in typical cyclic animals at greater frequency (46.67%) than that in acyclic animals (13.33%). In addition, *CC* genotype is present with high frequency in acyclic animals (73.33%) but in typical cyclic animals is occurred 40%. Kumar et al. (2009) found that thecontrol animals have the *TT*homozygous, but *TT* is substituted with the *TC*heterozygous in the same locationin animals that are fully developed and true anestrus with completely absence of *CC* homozygous. The difference may be attributed to number of animals selected for study, age difference, population size and the breed difference.

The *fshr* gene is composed of 10 exons and 9 introns (Simoni et al., 1997). FSH regulates ovarian follicle growth, differentiation, maturation, and ovulation via attaching to a specific receptor on the surface of the ovary (*fshr*) (Yang et al., 2012).
The present study showed that the amplified DNA fragment of *fshr* gene exon 10 has a polymorphic heterozygous change in all animals of the three groups (smooth inactive ovaries, silent heat and control) which genotyped as *GA* at 150, 200 bp except, one animal from silent heat group showed no polymorphism but monomorphic homozygous pattern genotyped as *AA* at 230 bp. The findings are consistent with those reported by ShafiK et al. (2017) who used the PCR-SSCP method to amplify the same region of current study in the *fshr* exon 10 gene in Egyptian buffaloes and found a non-synonymous G/A SNP resulting in a change of methionine into isoleucine amino acid.Marson et al. (2008) found that three genotypes (*GG, CG,* and *CC*) for the *fshr* gene existed.Additionally, Ahmed et al. (2011) reported that SSCP analysis revealed polymorphism in the fshr gene locus with a size of 306 bp. In contrast to our result, polymorphism didn't exist in the *fshr* exon 10 gene in Egyptian buffaloes (Othman and Abdel-Samad 2013; Sosa et al., 2015) and Murrah Buffaloes (Kathiravan et al., 2019). Furthermore, using the RFLP approach on Iraqi buffaloes, no polymorphism was found in the amplified 306 bp region of the *fshr* exon 10 gene (Al-Hamedawi et al., 2017). The difference may be attributed to inbreeding rate or the relationships between some of the sampled animals or the number of animals selected for studying.The three groups of this study show that the frequency of *A* allele was 182 located at 150, 200, 230 bp while, the *G* allele was 178 located at 150, 200 bp, with the same results obtained by Campagnani (2008) who recorded *GG* 0.49% in Brazilian buffaloes as well as Marison et al. (2008) recorded 0.3% of *GG* in Europe buffaloes.

CONCLUSION

There is no association between genetic polymorphism of both *cyp19* and *fshr* and reproductive disorders especially ovarian inactivity and silent heat in Egyptian buffaloes because almost animals were found homozygous (*GG*) for *cyp19*/PvuII and polymorphic heterozygous *(GA)* genotypes for *fshr*/Hin1II (NlaIII) locus but, research into polymorphisms and their interactions with reproductive issues is necessary.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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