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Genetic Polymorphism of Luteinizing Hormone Receptor Gene in Relation to Fertility of Egyptian Buffalo

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Abstract

The aim of this study was to detect the genetic polymorphism of luteinizing hormone receptor (LHR) gene and its association with fertility in Egyptian buffalo using PCR-RFLP and sequencing analysis. A total number of 301 comprised of 100 fertile and 201 non-fertile buffalo were used. Gynecological examinations (aided by ultrasonography) were carried out twice for two successive weeks. The genomic DNA was extracted from females and the PCR product was assessed. The percentages of inactive ovary and endometritis (grad, I) were the most common reproductive problems. The mean uterine horn diameter was largest during follicular phase followed by luteal phase and inactive ovary (2.67 ± 0.11, 2.28 ± 0.09 and 2.18 ± 0.11 cm, respectively). The PCR product of the primer specific for LHR gene gave the specific band at size 303 bp.The amplified fragments remained undigested by HhaI restriction enzymes and all tested buffalo were genotyped as TT. DNA sequence of 259 bp out of the amplified 303 bp was determined. The sequence alignment of 259 bp of Egyptian buffalo LHR with published sequence (accession number: XM_006078413.1 and JQ885687.1, Bubalus bubalis) showed that the 259 bp segment possess identities at 100%.

In conclusion, monomorphic pattern for LHR gene is considered a unique feature that may be related to the characteristic species in buffalo. Therefore, the polymorphisms and interaction with the fertility trait should be the subject for further research with a bigger number of animals.

Keywords: Buffalo; Fertility; Ultrasound; LHR; PCR-RFLP; Nucleotide sequences

Introduction

Productivity of buffalo is still low and there is a plenty of room for increasing productivity of buffalo through improvement of reproductive performance. The productivity of buffalo is considerably affected by inherent problems such as low reproductive efficiency, which is mainly due to late maturity, poor expression of estrus, anestrus, inactive ovaries, long postpartum interval, seasonality in cyclicity and silent estrus. Anestrus due to ovarian dysfunction or silent ovulation and repeat breeding are two major reproductive disorders in buffalo [1]. Regulation of reproductive function involves the hypothalamus–pituitary–gonad axis and its interactions. The follicular stimulating hormone (FSH) and luteinizing hormone

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(LH) receptor (FSHR and LHR) genes express at the gonads [2], being all of the members of the G protein coupled receptor family [3]. The gonadotropin luteinizing hormone (LH) plays a role in follicular development [4], ovulation [5,6], corpora lutea formation [7], and pre-implantation embryonic development [8,9]. The LH surge triggers ovulation and induces in granulosa cells of preovulatory follicles, the rapid and transient expression of certain genes in a species manner that have been shown to be critical for ovulation [10].

The cellular actions of LH are mainly mediated by the luteinizing hormone/choriogonadotropin receptor (LHCGR), which has features typical of receptors that interact with G proteins, including a cellular domain, seven transmembrane domains, and an extracellular hormone-binding domain [11]. As LH interacts with LHR, it influences various activities such as steroidogenesis, follicular growth, oocyte maturation, ovulation and corpus luteum formation, which are essential for reproductive function of the females [12]. Therefore, under physiologic conditions the appearance of LHR on granulosa cells is fundamental for folliculogenesis from the acquisition of follicular dominance until ovulation [13,14]. Despite the accumulation of considerable amounts of information about LHR [15], there is no report on genetic Polymorphism of LHR gene in fertile and infertile buffalo. The objective of this study was to investigate genetic polymorphism of LHR gene in fertile and infertile buffalo and its association with ovarian status in different fertility problems in Egyptian buffalo.

Materials and Methods

The present study was conducted, using a total number of 301, comprised of 100 fertile and 201 non-fertile buffalo with the history of anestrum and repeat breeder reared in Meet Kenana village, Kalubia Province. The animals were brought to a veterinary clinic for treatment of infertility problems and confirming of pregnancy in the fertile animals during different season of 2013. A full case history and owner explanation of each animal were recorded. Gynecological examinations were carried out twice for two successive weeks to identify the reproductive status and/or disorder.

Ultrasonographic Examination

Ultrasonographic examination of the buffalo was conducted prior to blood sampling through a transrectal ultrasonography with a B-mode scanner (Magic 2200, Eickemeyer Veterinary Equipment Inc., Germany) equipped with 6 MHz linear-array transducer for identifying the state of follicular growth and the presence or absence of corpus luteum on the ovary. The transverse diameter of the anterior 1/3 section of both uterine horns was evaluated according to Kandiel et al. [16].

Blood Sampling and DNA Isolation

Blood sampling was carried out on the animal using EDTA anticoagulant vacutainer tubes. Genomic DNA was extracted from blood samples using the Relia prep^{TM} DNA blood kit (Promega, USA) according to the manufacturer's instructions.

PCR Reaction and DNA Amplification

The genetic polymorphism was analyzed using primers representing exon 1 L in LHR gene. Amplification reactions were done in a final volume of 50 µL, containing 5 µL buffer 10x , 1 µL 2.5 mM (dNTPs mixture), 3 µl 25 mM ($MgCl_2$), 0.25 µL primer 1, 0.25 µL primer 2), 0.3 µL Taq polymerase (5 U/µL), 35.2 µL water (nuclease free water), 5 µL DNA sample. The PCR reactions were as follow: one cycle at 95°C for 60 s (initial denaturation), followed by 30 cycles of 94°C for 60 s, 60°C

for 60 s and 72°C for 60 s (Table 1). After the reactions, the PCR products were subjected to electrophoresis in a 2.5% agarose gel, at 60 V for approximately 2.5 h. The size of the amplified product was compared using the 50 bp DNA ladder.

Primer	**Primer sequence (5' – 3')**	**PCR condition**	**PCR product size (bp)**	**Restriction enzyme**	**References**
Forward Reverse	CAAACTGACAGTCCCCCGCTTT CCTCCGAGCATGACTGGAATGGC	94°C 1 min 58°C 45 s 72°C 1 min	303 bp	HhaI	[17]

Table 1: Primer sequence and properties used for amplification of LHR gene.

Restriction Fragment Length Polymorphism (RFLP) Technique

For genotyping, the PCR products were digested with HhaI. Gene fragments was subjected to digestion by restriction enzymes in a total volume of 20 µL (10 µL reaction solution, 2 µL enzyme buffers, 0.2 µL enzymes, and 7.8 µL water) and placed in the thermocycler at 37°C for 1 h.

Sequence Analysis

The PCR product was purified using QIAquick PCR purification kit (QIAGEN) and PCR purification spin protocol (QIAGEN), designed for the isolation of DNA fragments from PCR reactions. The PCR products were sequenced by Macrogen Incorporation (Seoul, South Korea). Sequence analysis and alignment were carried out using NCBI/BLAST/blastn suite.

Statistical Analysis

The obtained data (mean ± SEM) was tabulated and statistically computed by one way analysis of variance (ANOVA) using SPSS (Statistical Package for the Social Sciences) program Ver. 16. The incidence of animals had dominant (F1) and/or largest (F2) follicles on the same or different ovaries was tested using Chi-square analysis. The level of significance was set at $p < 0.05$.

Results

As is presented in Table 2, more animals were brought to the veterinary clinics in the summer than those in other seasons were.

The total percentage of anestrus and repeat breeder animals was 48.2 and 18.6 respectively. The percentage of anestrum animal decreased with increasing parity number, while the percentage of repeat breeders increased with increasing the parity number.

Season	Pregnant	Anestrum				Repeat breeder				Total No. (%)
		Heifers	Multiparous			Heifers	Multiparous			
			1st parity	2nd parity	≥ 3rd parity		1st parity	2nd parity	≥ 3rd parity	
Summer	49	7	23	18	13	6	5	6	10	137 (45.5)
Spring	34	6	16	5	10	2	3	4	8	88 (29.2)
Autumn	10	1	14	6	3	2	1	2	1	40 (13.2)
Winter	7	1	12	6	4	2	1	1	2	36 (11.9)
Total, No. (%)	100 (33.22)	15 (4.98)	65 (21.59)	35 (11.63)	30 (9.97)	12 (3.97)	10 (3.32)	13 (4.32)	21 (6.98)	301
		(48.2)				(18.6)				

Table 2: Number and percentage of fertile and infertile buffalo examined during different season of the year.

Incidence of various reproductive disorders investigated by ultrasonography in Egyptian buffalo was presented in Table 3. The proportion of normal animals was 170 out of 301 (56.48%). Different pathological conditions affect buffalo's fertility has been observed, with the highest incidence of ovarian inactivity (7.97%) and endometritis grade I (7.64%) were encountered among ovarian and uterine disorders, respectively.

Reproductive state and/or disorder	Number	Percentage (%)
Fertile animals		
- Non-pregnant: Follicular phase	50	16.61
Luteal phase	48	15.95
- Pregnant	72	23.92
Infertile animals		
- Abnormal ovarian condition and normal uterus		
Inactive ovary	24	7.97
Bilateral smooth ovary	13	4.32
Unilateral smooth ovary	5	1.66
Follicular cyst	1	0.33
Ovarian tumor	1	0.33
- Normal ovarian condition and abnormal uterus		
Endometritis grade I	23	7.64
Endometritis grade II	13	4.32
Endometritis grade III	10	3.32
Uterine adhesions	7	2.33
Hydrometra	2	0.66
- Abnormal ovarian and uterine conditions	32	10.63
Total	301	100

Table 3: Ultrasonographic surveying of reproductive disorders in Egyptian buffaloes.

A representative images of ovaries from Egyptian buffalo suffered from smooth (A, B) or inactive (C, D) ovaries was illustrated in Figure 1. The number of animals having only one dominant follicle per two ovaries were significantly higher ($P \leq 0.001$) during luteal phase than that in follicular phase or inactive ovaries. Animals had dominant and second largest follicle either on the same ovary or both ovaries tended to be higher (P=0.09) during follicular phase. The total number of follicles per two ovaries per animal and the mean diameter of dominant were significantly (P<0.001) high in absence of corpus luteum. On the other hand, ovarian inactivity was characterized by smaller diameter of dominant and 2nd largest follicles. The difference between the dominant and second largest follicle size was significantly (P<0.001) more in animals during follicular phase (0.51 ± 0.04 cm), and less in inactive ovary (0.19 ± 0.03 cm). The mean uterine horn diameter was largest during follicular phase followed by luteal phase and inactive ovary (2.67 ± 0.11, 2.28 ± 0.09 and 2.18 ± 0.11 cm, respectively).

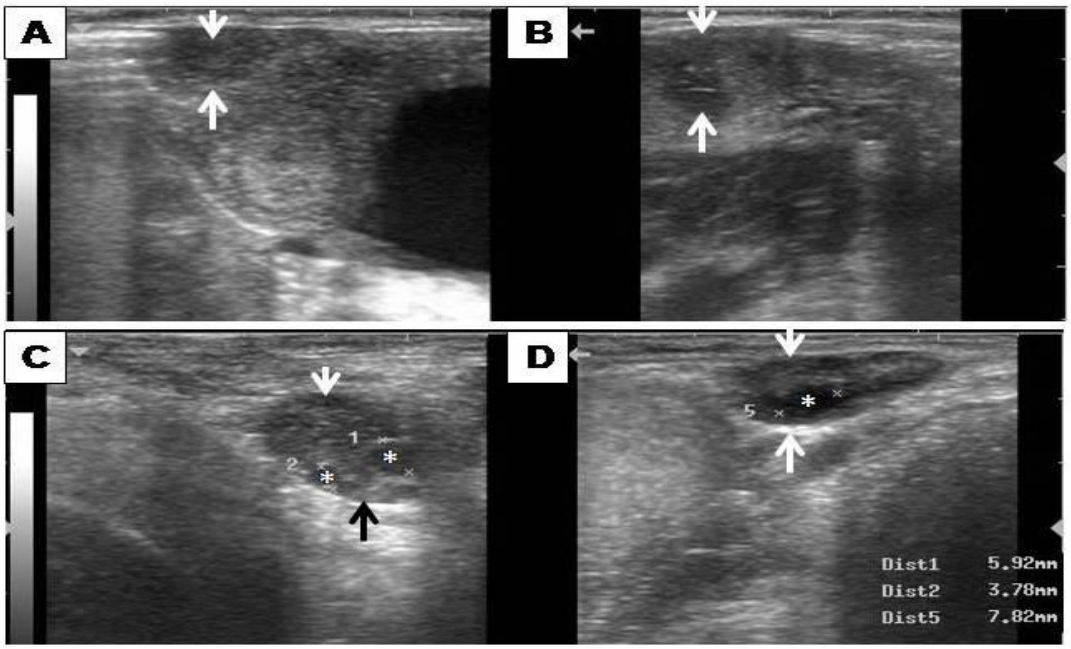

FIG.1: Ultrasonographic image of representative ovaries from buffaloes suffered from smooth (A, B) or inactive (C, D) ovarian inactivity. Arrows and stars indicated the ovarian position and follicles, respectively.

The 303 bp PCR product of the LHR gene was digested using restriction endonuclease HhaI followed by agarose gel electrophoresis (Figure 2). The 303 bp PCR product remained undigested by HhaI restriction enzyme (Figure 3).

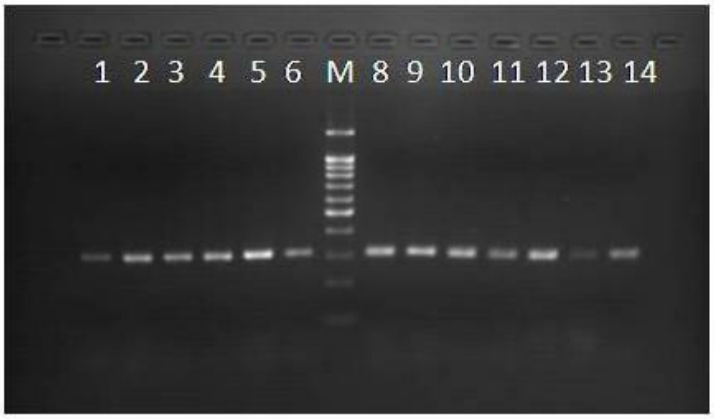

FIG.2: DNA electrophoretic pattern was obtained after PCR amplified with buffalo LHR gene (lane, 1-6 and 8-14). M 100 bp ladder marker.

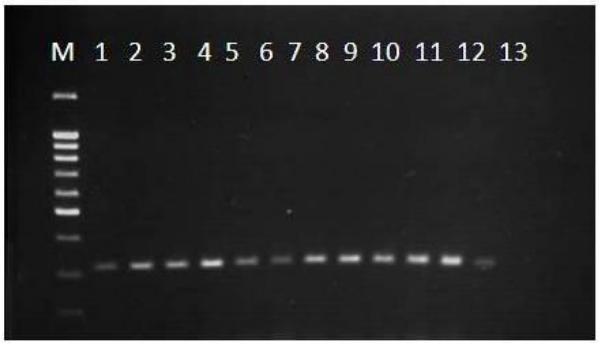

FIG. 3: DNA electrophoretic pattern was obtained after digestion of PCR amplified buffalo of LHR gene product with HhaI. M 100 bp ladder marker, Lanes from 1-12 showed undigested band (303 bp).

A 259 bp out of the 303 bp PCR- product was sequenced , and was aligned with published sequence (accession number: XM_006078413.1, *Bubalus bubalis*) using BLAST. The results showed that the 259 bp segments possess identities at 100% (Figure 4). The result also revealed that the sequence of the same DNA segment is 100% identities with the accession number: GenBank: JQ885687.1, *Bubalus bubalis* (Figure 5).

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Query  3     GACTTCTGCATGGGACTCTACCTGCTGCTCATTGCCTCAGTTGATGCCCAGACCAAAGGC  62
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1366  GACTTCTGCATGGGACTCTACCTGCTGCTCATTGCCTCAGTTGATGCCCAGACCAAAGGC  1425
Query  63    CAGTATTACAACCATGCCATAGACTGGCAGACAGGGAGTGGGTGCAGCACGGCTGGCTTT  122
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1426  CAGTATTACAACCATGCCATAGACTGGCAGACAGGGAGTGGGTGCAGCACGGCTGGCTTT  1485
Query  123   TTCACTGTGTTTGCAAGTGAACTCTCTGTCTACACCCTCACAGTCATCACACTAGAAAGA  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1486  TTCACTGTGTTTGCAAGTGAACTCTCTGTCTACACCCTCACAGTCATCACACTAGAAAGA  1545
Query  183   TGGCACACCATCACCTATGCTATTCAACTGGACCAAAAGCTGCGACTGAAACATGCCATT  242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1546  TGGCACACCATCACCTATGCTATTCAACTGGACCAAAAGCTGCGACTGAAACATGCCATT  1605
Query  243   CCAGTCATGCTCGGAGG  259
             |||||||||||||||||
Sbjct  1606  CCAGTCATGCTCGGAGG  1622
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Figure 4: sequence analysis of 259 segment of Egyptian buffalo LHR amplified product compared to buffalo accession numberXM_006078413.1

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Query  3     GACTTCTGCATGGGACTCTACCTGCTGCTCATTGCCTCAGTTGATGCCCAGACCAAAGGC  62
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  47    GACTTCTGCATGGGACTCTACCTGCTGCTCATTGCCTCAGTTGATGCCCAGACCAAAGGC  106
Query  63    CAGTATTACAACCATGCCATAGACTGGCAGACAGGGAGTGGGTGCAGCACGGCTGGCTTT  122
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  107   CAGTATTACAACCATGCCATAGACTGGCAGACAGGGAGTGGGTGCAGCACGGCTGGCTTT  166
Query  123   TTCACTGTGTTTGCAAGTGAACTCTCTGTCTACACCCTCACAGTCATCACACTAGAAAGA  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  167   TTCACTGTGTTTGCAAGTGAACTCTCTGTCTACACCCTCACAGTCATCACACTAGAAAGA  226
Query  183   TGGCACACCATCACCTATGCTATTCAACTGGACCAAAAGCTGCGACTGAAACATGCCATT  242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  227   TGGCACACCATCACCTATGCTATTCAACTGGACCAAAAGCTGCGACTGAAACATGCCATT  286
Query  243   CCAGTCATGCTCGGAGG  259
             |||||||||||||||||
Sbjct  287   CCAGTCATGCTCGGAGG  303
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Figure 5: sequence analysis of 259 segment of Egyptian buffalo LHR amplified product compared to buffalo accession number JQ885687.1

Discussion

There are considerable interests in identifying genes involved in the regulation of reproductive traits. The reproductive function is a major factor determining the economic importance of buffalo [18]. This study showed that more animals were brought to the veterinary clinics in the summer than those in other seasons.

Kumar et al. [19] reported that incidence of anestrus in buffalo is severe during summer. The total percentages of anestrus and repeat breeder animals were 48.2 and 18.6, respectively. It was reported that anestrus is the most important cause of poor reproductive performance in buffalo [20]. This study revealed that, the number of animals with anestrum decreased with increasing parity number. Ali and El–Sheikh [21] reported longer postpartum anestrus in primiparous than that in multiparous buffalo. Moreover, Mahdy et al. [22] reported that as the parity number increases, the postpartum anestrus period decreases.

The present work showed that the number of repeat breeder animal was increased with increasing the parity number which is in accordance with findings of Bonneville-Hébert et al. [23]. However, Kumar et al. [24] reported that the highest incidence of repeat breeding was observed in second parity and lowest incidence was observed in the forth parity and onward parity (11.11%). The incidence of abnormalities of the reproductive tract of buffalo in the current study was 131 out of 301 (43.52%). This is lower than that reported in former study [25]. These variations could be attributed to the efficacy and specificity of ultrasonography in diagnosis of infertility problems as compared with gross examination or rectal palpation.

Inactive or non-functional ovary is the most important reason affecting the estrus behavior and characterized by an inadequate follicular development and absence of ovulatory follicle. In the current study, the incidence of ovarian inactivity as well as endometritis grade I was the highest among the recorded reproductive disorders (7.97 and 7.64%, respectively). In this respect, Gad [26] stated that endometritis was the most common affection in cows and buffalo, while other causes of uterine affections were relatively lower in their incidence.

The current investigation clearly demonstrated the controlling role of CL on ovarian activity in buffalo where the number of animals had only one dominant follicle per two ovaries was significantly higher during luteal phase. Besides, in absence of CL, the total number of follicles per two ovaries per animal and the mean diameter of dominant were significantly ($P<0.001$) high. The presence of CL is responsible for atresia of the largest follicle due to the negative feedback effect of progesterone secretion on pituitary release of LH. Nevertheless, during the luteolysis process, the largest follicle starts to increase in diameter progressively and finally leads to ovulation. Yilmaz et al. [27] showed that the mean diameter of the largest follicle as well as the number of follicles at above 12 mm in diameter were substantially higher in follicular phase than that in other stages of estrous cycle in buffalo. The number of dominant follicles that developed in the ovary ipsilateral to the CL was greater than in the contralateral ovary [28].

Functional ovarian inactivity; characterized by lacking of ovulation (no CL) and absence of large follicles (>10 mm in diameter); is one of the main causes of sub estrus or anestrus and may limit the breeding value of buffalo. In the current study, this condition was characterized by smaller diameter of both dominant and the 2^{nd} largest follicles and the difference between the dominant and 2^{nd} largest follicle was considerably small. The later parameter could be used to demark it from an active ovary, as the deviation in the growth between the dominant and the 2^{nd} largest follicle was great during follicular phase possibly due to high rate of atresia of small follicles. In vitro study showed that FSH stimulates growth rate, steroidogenesis, and DNA synthesis of buffalo preantral follicles, though the development to the dominant follicle suppresses further growth of smaller follicles [28]. Basal concentrations of LH is important for promoting the maturation of ovarian follicles, stimulating the preovulatory steroidogenic changes [28] until high and pulsatile production of LH determine the dehiscence of the mature follicle and ovulation. This suggests that the estrogen-dominant follicular phase boosts follicular growth, due presumably to its positive feedback on gonadotrophins (mainly LH).

The fitness of tubular part of female reproductive system is essential for optimal reproductive performance of the animal. The changes therein due to various physiological or pathological conditions may affect the functional status. The current study demonstrated that the mean uterine horn diameter was largest during follicular phase followed by luteal phase and inactive ovary (2.67 ± 0.11, 2.28 ± 0.09 and 2.18 ± 0.11 cm, respectively). The pattern of uterine measures during estrous cycle is

consonant with Honparkhe et al. [29] who used palpation per rectum as well as ultrasonography to establish the size of reproductive organs of cycling buffalo. The increased uterine horn size during follicular phase might be due to increased vascular development and edema resulted from high estradiol concentration from large developing follicles. On the other hand, the small uterine horn diameter in inactive ovarian conditions might be due to the low estrogenic activity of ovarian structures, which were generally small in diameter.

In the present study, the primers for LHR gene was amplified DNA fragments with sizes 303 bp. This result is in agreement with Othman and Abdel-Samad [30] in buffalo. The use of DNA polymorphic markers allows the determination of individual genotypes at any loci and provides information on allele frequencies as well as improving selection by marker assisted selection. In this study, PCR-RFLP technique was used to identify polymorphic regions in a fragment of the LHR gene in buffalo. The results demonstrated no polymorphic region in this fragment which in accordance with that found by Othman and AbdeL-Samad [30] in buffalo, Marson et al. [31] 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. Higher values for TT (0.540) and lower values for CC (0.030) were reported by Milazzotto [32] for a Nellore population. Yu et al. [6] concluded that, heifers with the GG and GT genotypes had a higher total number of ova than those with the TT genotype, and heifers with the GG genotype had a significantly higher number of transferable embryos than those with GT and TT. The absence of other alleles than TT allele in our study may be attributed to the lower number of animals comparing with other studies and due to genetic background of the buffalo.

Conclusion

Monomorphic pattern of TT allele for LHR gene is considered a unique feature that may be related to the characteristic species in buffalo. Therefore, the polymorphisms and interaction with the fertility trait should be the subject of further research using a bigger number of buffalo.

Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgement

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