



Detection of SNPs in Exon10 Locus of FSHR Gene and Their Association with Some Infertility problems in Egyptian Buffaloes

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ABSTRACT

Follicle-stimulating hormone (FSH) is a glycoprotein hormone. It helps in development and maturation of the follicles in females through binding to FSH receptors which consists of 10 exons and 9 introns. The objective of this study was to estimate some genetic factors affecting milk production and fertility traits and to detect polymorphisms in exon 10 of FSHR gene and determine the associations between these polymorphisms and infertility in Egyptian buffalo. Heritability estimates were (0.19, 0.18, 0.07, 0.10, 0.21 and 0.19) for calving interval, days open, dry period, days in milk, total milk yield and 305day milk yield (305 DMY), respectively. Dry period showed high positive phenotypic and genetic correlation with day's open and calving interval. The breeding value for 305 DMY ranged from -480 to 380, from -370 to 330 and between -230 to 170 kg for cow, sire and dam, respectively. There was one non synonymous SNP (A93G) at 93bp in exon 10 of FSHR gene (with 230bp size) and there was two different pattern of single strand conformation polymorphism (SSCP) but there was no any SNP for FSHR gene (with 306bp size) in all examined Egyptian buffaloes as shown by one SSCP pattern and nucleotide sequencing.

Keywords: correlation. Egyptian buffaloes. FSHR. heritability. Polymorphism. SNP. SSCP.

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1. INTRODUCTION

River buffaloes are the main source of meat and milk production in Egypt so it considers of a great economic value. Genetic improvement of productive traits of buffaloes has been dependent on some information of quantitative genetics of various traits of economic importance to make efficient and easy selection of Egyptian buffaloes (Kumar et al., 2009). Anestrous due to ovarian dysfunction and silent heat also repeat breeding are two major reproductive disorders in buffaloes (Goley and Kadu, 1995). The major cause for low productivity in buffalo is poor reproductive efficiency that is caused by delayed maturity, silent estrous, poor expression of estrous, anestrous and irregular estrous cycle (Sana et al., 2014). Reproductive efficiency has high priority in all breeding systems and for its understanding need more studies especially in poor and seasonal breeder animals. Genetics is the main way for the

selection of superior animals that can transmit their favorable traits to their progeny (Emtenan et al., 2014). The endocrine system is the most regulators of the reproductive functions of the body through interactions between the hypothalamus, pituitary gland and gonad. The Follicle Stimulating Hormone Receptor (FSHR) gene is expressed in the gonads. The actions of Follicle Stimulating Hormone (FSH) on ovarian and testicular somatic cells through binding with these receptors (Themmen and Huhtaniemi, 2000). FSH is fundamental for sertoli cell function which stimulate spermatogenesis and growth of the follicles by binding with its specific receptor (FSHR) (Zalata et al., 2008).

The objective of this study was: A. Estimation of some genetic parameters as heritability, genetic and phenotypic correlations among some traits and estimate expected breeding values. B.

Identification of polymorphisms in the candidate gene (FSHR) associated with some reproductive problems in Egyptian buffaloes.

2. MATERIAL AND METHODS

The data used in the present study were collected from the productive and reproductive records of Egyptian buffaloes maintained at Mahalet-Mousa experimental farms of Animal Production Research Institute (APRI), Agricultural Research Centre, Ministry of Agriculture. By rectal examination and ultrasonography, animals were divided into three groups: animals were cyclic, animals having anoestrous (due to ovarian inactivity) and animals which had more than three services (repeat breeder animals).

2.1. DNA extraction:

Under investigation, blood samples were taken from 60 selected animals from jugular veins in EDTA- containing vacutainer tubes, kept in ice box and stored at stored at -20°C till further processing. The genomic DNA was extracted from the whole blood by using TIANamp Genomic DNA Kit (www.tiangen.com/en). The concentration of DNA was measured by using Nanodrop. The experiments were carried out at Central Lab, Faculty of Veterinary Medicine, Benha University, Egypt.

2.2. Polymerase chain reaction (PCR):

The Exon 10 of FSHR locus was amplified by PCR using primers (Table 1). PCR was performed by employing a PCR program as follows: - Initial denaturation step at 94°C for 5 min, then tubes were subjected to 30 cycles of 94°C for 1 min, 57°C for 2 min and 72°C for 2 min for FSHR (with size 230bp), 62°C for 1 min and 72°C for 1 min for FSHR (with size 306bp) followed by a final extension step at 72°C for 10 min. Then PCR products were resolved by electrophoresis and stained with ethidium bromide and visualized with UV light of Gel Documentation System.

2.3. Single Strand Conformation Polymorphism (SSCP):

5 μL PCR products were mixed well with 5 μL formamide loading dye then heated in thermal cycler for 10 minutes at 98°C then chilled on ice. This product loaded on non-denaturing 10% polyacrylamide gel and electrophoresis was performed in 1 \times TBE buffer at 150 V for 90 minutes. After electrophoresis: gel was immersed in a tray filled with 250ml of ethidium bromide (25 $\mu\text{g}/500\text{ ml}$ in 1 \times TBE) for 20 minutes then

transferred gently in 200ml of distilled deionized water for de-staining for 5 minutes and finally the fragments patterns were photographed by the gel documentation system (UVDI Major Science, USA).

2.4. DNA Sequencing:

After getting purified PCR products with expected size by using PCR purification Zymoclean™ Gel DNA Recovery Kit to remove primers, primer dimmers, salt, proteins, ethidium bromide and agarose. The sequencing was done by using forward primer in 3500 Genetic Analyzers (Applied Biosystems®). BLAST software (<http://www.ncbi.nlm.nih.gov/>) was used for sequence identification and confirmation. The MEGA 6 (Kumar et al., 2008), Finch TV 1.4.0 (<http://www.geospiza.com/finchtv/>) and Bioedit 7.0.5.3 (Hall, 1999) software were used for sequences alignment and polymorphism detection.

2.5. Statistical Data Analysis:

2.5.1. Heritability, Genetic correlation and breeding value:

Heritability, genetic correlation and breeding value of studied traits were estimated with derivative free restricted maximum likelihood (DFREML) procedures using (MTDFREML) program of (Boldman et al., 1995). The assumed model was: $Y = Xb + Za + e$ Where: - Y: is the vector of the observed trait, X: is the incidence matrix of fixed effects, b: is the vector of fixed effects, Z: is the incidence matrix of random animal effects, a: is the vector of random animal effects and e: is the vector of random residual effects.

2.5.2. Phenotypic correlation:

The phenotypic correlation between traits x and y is calculated by SAS (SAS, 2001).

2.5.3. Gene and Genotypic frequencies:

Gene and genotype frequencies of *FSHR* gene (with size 230bp) SNP in buffalo calculated by applying the χ^2 test. $P^2 + 2pq + q^2 = 1$ (Snedecor and Cochran, 1976).

3. RESULTS

Table (2) showed that days in milk, calving interval and days open, total milk yield and 305day milk yield had a moderate heritability estimates. High heritability estimates were for age at first service and calving. Age at first service showed negative phenotypic correlation with other traits except age at first calving. Dry period showed high

positive phenotypic and genetic correlation with day's open and calving interval Table (3).

Table (4) mentioned the estimated breeding values of sire, dam and cow. Expected breeding value average of 305day milk yield, calving interval and age at first service was higher in dam. While, Expected breeding value average of dry period and age at first calving was higher in cow. Moreover, expected breeding value average of days open, days in milk and total milk yield was higher in sire

The PCR product with the expected size (230bp and 306bp) for exon 10 of FSHR gene was observed on agarose gel electrophoresis (Figure 1 and 2). There is one non synonymous SNP (A93G) at 93bp (Figure 6) and led to change of methionine

amino acid into isoleucine amino acid in exon 10 of FSHR gene (with 230bp size) which identified by PCR-SSCP and sequencing in female Egyptian water buffaloes. There was two different pattern of PCR-SSCP for this gene (Figure 3). Nucleotide sequences alignment of FSHR gene showed 98% identity with *Bubalus bubalis* (EU148059.1) (Figure 7).

In exon 10 of FSHR gene (with size 306bp), there was no any SNP in all examined Egyptian buffaloes by sequencing (Figure 8) which shown by only one pattern SSCP (Figure 4). Nucleotide sequences alignment of FSHR gene showed 96% identity with *Bubalus bubalis* (EF560049.1) (Figure 9).

Table (1): Primer Information for Analysis:

Gene	Primer sequences (5' - 3')	PCR product	Reference
FSHR	F: ATCACGCTGGAAAGATGGCATACC R: GACATTGAGCACAAG GAGGGA C	230 bp	(Yong et al., 2006)
FSHR	F: CTGCCTCCCTCAAGGTGCCCTC R: AGTTCTGGCTAAATGTCTTAGGGG	306 bp	(Othman and Abdel-Samad, 2013)

Table (2): Heritability Estimates among Different Studied Milk Production and Fertility Traits.

Trait	$h^2 \pm$ S.E
AFS	1.00 \pm 0.093(O.E)
AFC	0.98 \pm 0.001(O.E)
CI	0.19 \pm 0.036
DO	0.18 \pm 0.036
DP	0.07 \pm 0.037
DIM	0.10 \pm 0.028
TMY	0.21 \pm 0.035
305 DMY	0.19 \pm 0.035

O.E: overestimated.

Table (3): Phenotypic (above the diagonal) and Genetic Correlations (below the diagonal) among Different Milk Production and Fertility Traits.

Trait	AFS	AFC	CI	DO	DP	DIM	TMY	305DMY
AFS	-	0.02**	-0.03	-0.02	-0.05	-0.02	-0.01	-0.03
AFC	0.02	-	0.09**	0.10**	0.06	-0.06	-0.11**	-0.06
CI	-0.17	-0.23	-	0.97**	0.88**	-0.007	0.004	0.02
DO	0.01	-0.03	1.00	-	0.86**	-0.008	0.008	0.03
DP	0.05	0.04	0.96	0.97	-	-0.05	-0.09*	-0.03
DIM	0.67	-0.13	0.56	0.55	-0.50	-	0.46**	-0.15**
TMY	0.07	-0.22	0.38	0.29	-0.23	0.73	-	0.69**
305DMY	0.09	-0.13	0.18	0.25	0.16	0.29	0.86	-

Table (4): Breeding Value Estimates for Different Studied Milk Production Traits and Fertility Traits for Cow, Sire and Dam.

Trait	Estimated Breeding Value								
	Cow			Sire			Dam		
	Min	Max	Average	Min	Max	Average	Min	Max	Average
AFS	-7.05	11.95	-0.36	-4.7	5.6	-0.26	-3.8	7.2	-0.1
AFC	-15.8	25.2	0.26	-7.9	11.4	0.12	-7.9	16.5	0.07
CI	-1.6	3.7	-0.01	-0.9	1.9	0.004	-1.5	2.4	0.002
DO	-43.1	97.9	-0.21	-41.9	76.3	0.08	-29.4	51.8	0.05
DP	-24	44.9	0.07	-15.4	22.5	-0.12	-17.6	22.5	0.02
DIM	-20.2	32.8	-0.51	-12.3	18.3	0.15	-10.6	15.8	0.03
TMY	-380	370	-10	-310	240	2	-160	180	0.5
305DMY	-480	330	-20	-370	330	-0.4	-230	170	0.1

Figure (1): Stained agarose gel of PCR products with Eethidium bromide representing amplification of FSHR gene (exon10) with size of 230 bp (lanes1-6) in Egyptian buffaloes. M represents 100 bp ladder.

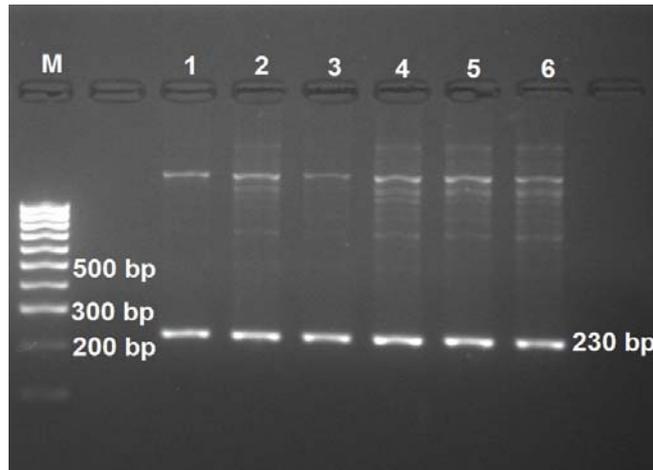


Figure (2): Stained agarose gel of PCR products with ethidium bromide representing amplification of FSHR gene (exon10) with size of 306 bp (lanes1-6) in Egyptian buffaloes. M represents 100 bp ladder.

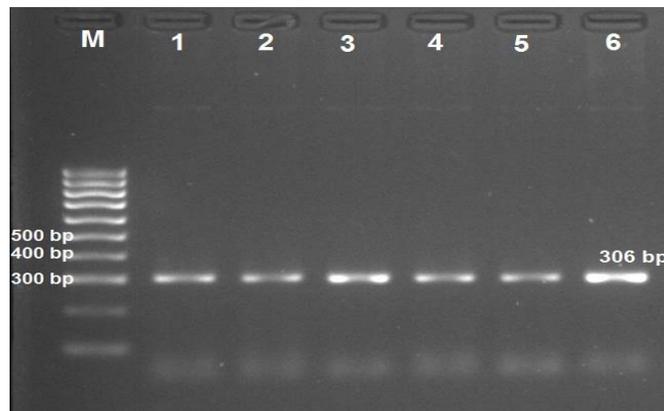


Figure (3): PCR-SSCP patterns of FSHR gene exon 10 with size 230 bp in Egyptian buffaloes. Two SSCP patterns were detected in FSHR gene; genotype GG (lane 1-6) and genotype AG (lane 7).

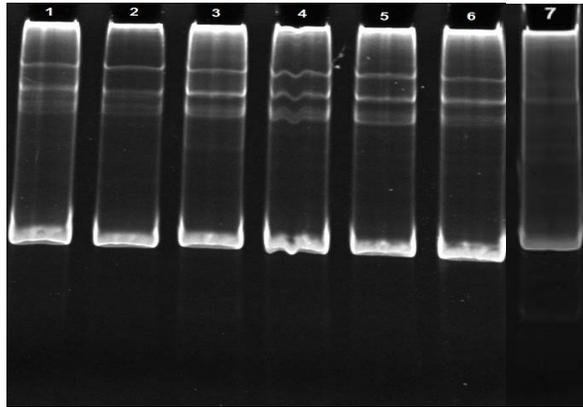


Figure (4): PCR-SSCP patterns of FSHR gene exon 10 with size 306 bp in Egyptian buffaloes. One PCR-SSCP pattern (monomorphic) was showed.

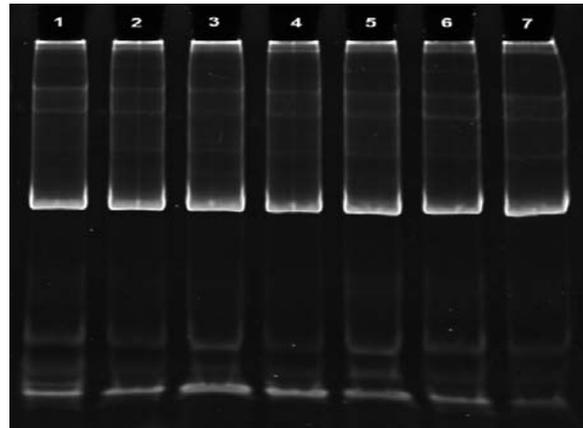


Figure (5): Nucleotide sequences of FSHR in Egyptian water buffaloes showed A93G SNP at nucleotide number 93 of exon 10 (with size 230bp).

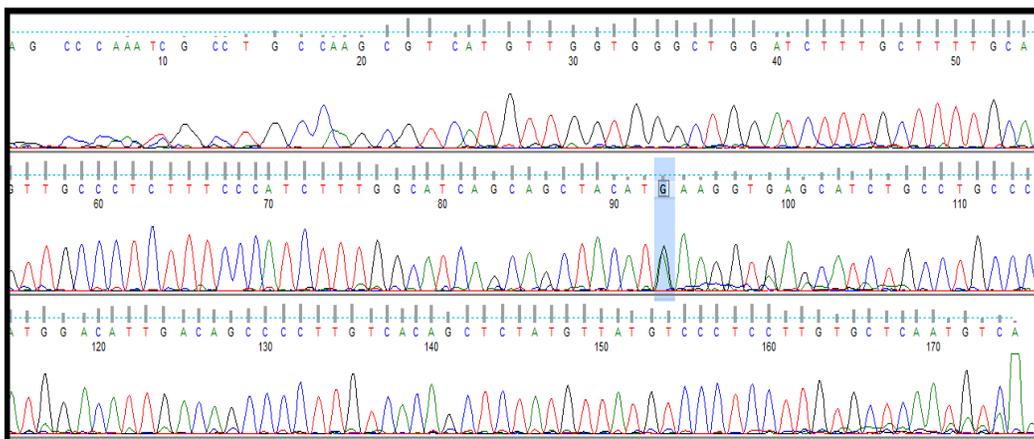


Figure (6): The sequence of FSHR gene exon 10 (with size 230bp) showed one non synonymous SNP (A93G).

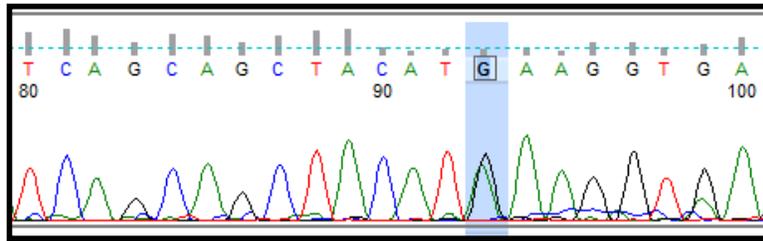


Figure (7): Nucleotide sequences alignment of FSHR (exon10) with size 230 bp using BLAST with Bubalus bubalis FSHR (exon10) (EU148059.1) showed 98% identity.

Bubalus bubalis follicle stimulating hormone receptor (FSHR) gene, exon 10 and partial cds
 Sequence ID: [EU148059.1](#) Length: 1234 Number of Matches: 1

Range 1: 533 to 763 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
399 bits(216)	1e-107	226/231(98%)	0/231(0%)	Plus/Plus
Query 1	ATCACGCTGGAAAGATGGCATACCAITCACCCATGCCATGCAGCTCGAATGCAAAGTGCAG	60		
Sbjct 533	ATCACGCTGGAAAGGTGGCATACCAITCACCCATGCCATGCAGCTCGAATGCAAAGTGCAG	592		
Query 61	CTCCGCCATGCTGCCAGCAICATGCTGGTGGGCTGGATCTTTGCTTTTGCAGTTGCCCTC	120		
Sbjct 593	CTCCGCCATGCTGCCAGTGTGCTGCTGGTGGGCTGGATCTTTGCTTTTGCAGTTGCCCTC	652		
Query 121	TTTCCCAICTTTGGCAICAGCAGCTACATAAAGGTGAGCAICTGCCCTGCCCATGGACATT	180		
Sbjct 653	TTTCCCAICTTTGGCAICAGCAGCTACATGAAGGTGAGCAICTGCCCTGCCCATGGACATT	712		
Query 181	GACAGCCCTTGTACAGCTCTAIGTTAIGTCCCTCCTTGTGCTCAAATGTC	231		
Sbjct 713	GACAGCCCTTGTACAGCTCTAIGTTAIGTCCCTCCTTGTGCTCAAATGTC	763		

Table (4): Genotype and allelic frequencies at A93G SNP of buffalo FSHR gene and estimated by Chi-Square (χ^2) test.

SNP	Genotype frequencies			Allele frequencies		P value
	AA	AG	GG	A	G	
A93G	0.02%	2.82%	97.16%	1.43%	98.57%	0.9317

Figure (8): Nucleotide sequences of FSHR exon 10 (with 306bp) in Egyptian water buffaloes showed no polymorphism.

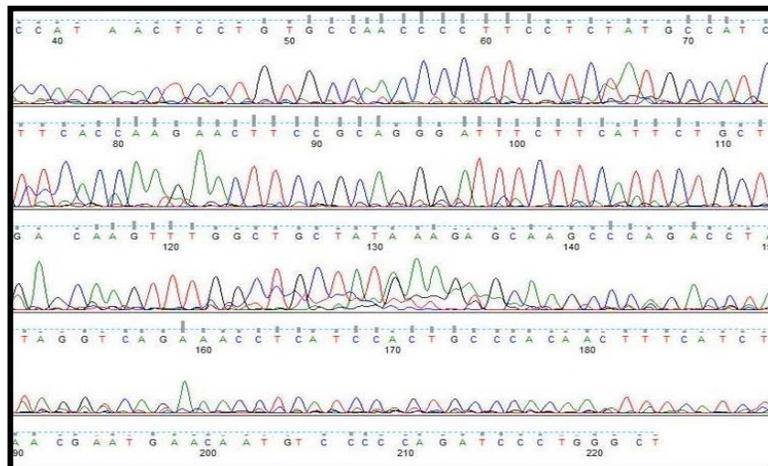


Figure (9): Nucleotide sequences alignment of FSHR (exon10) with size 306 bp using BLAST with Bubalus bubalis FSHR (exon10) (EF560049.1) showed 96% identity.

Bubalus bubalis follicle stimulating hormone receptor variant 7 gene, exon 10 and partial cds					
Sequence ID: EF560049.1 Length: 1233 Number of Matches: 1					
Range 1: 926 to 1231 GenBank Graphics ▼ Next Match ▲ Previous Match					
Score	Expect	Identities	Gaps	Strand	
505 bits(273)	3e-139	295/306(96%)	0/306(0%)	Plus/Plus	
Query 1	CTGCCTCCCTCAAGGTCGCCCTCATCACTGTGTCCAAGTCAAAGATCCTCCTGGTCCTAT				60
Sbjct 926	CTGCCTCCCTCAAGGTCGCCCTCATCACTGTGTCCAAGTCAAAGATCCTCCTGGTCCTAT				985
Query 61	TCTACCCCATCAACTCCTGTGCCAACCCCTTCCTCTATGCCATCTTCACCAAGAACTTCC				120
Sbjct 986	TCTACCCCATCAACTCCTGTGCCAACCCCTTCCTCTATGCCATCTTCACCAAGAACTTCC				1045
Query 121	GCAGGGATTTCITCATTCTGCTGAGCAAGTTTGGCTGCTATAAAGAGCAGCCAGACCT				180
Sbjct 1046	GCAGGGATTTCITCATTCTGCTGAGCAAGTTTGGCTGCTATAAAGAGCAGCCAGACCT				1105
Query 181	ATAGGTCAGAAACCTCATCCACTGCCCAACACTTTCATCTAACGAATGAACAATGTCCCC				240
Sbjct 1106	ATAGGTCAGAAACCTCATCCACTGCCCAACACTTTCATCTAACGAATGAACAATGTCCCC				1165
Query 241	CAGATCCCTGGGCTACCAGTGGTTCCAATTACACACTTATCCCCCTAAGACATTTAGCCA				300
Sbjct 1166	CAGATCCCTGGGCTACCAGTGGTTCCAATTACACACTTATCCCCCTAAGACATTTAGCCA				1225
Query 301	AGAACT 306				
Sbjct 1226	AGAACT 1231				

4. DISCUSSION

A low heritability estimate was 0.07 for dry period. The low heritability estimates were found in dry period indicated that the major part of the variation may be due to non-genetic factors and better management could play a good role in improving this trait. These results agreed with (Sujit and Sadana, 2000) and (Thevamanoharan et al., 2002). On the contrary to the present findings (Aziz et al., 2001), (Kawthar et al., 2005), (Thiruvankadan et al., 2010) and (Shalaby et al., 2013) who showed moderate heritability estimates for dry period. A moderate heritability estimates was 0.10, 0.21 and 0.19 for lactation length, total milk yield and 305day milk yield, respectively. The obtained results were in the same line of those obtained by (Thevamanoharan et al., 2002), (Mourad and Khattab, 2009), (El-Bramony, 2011), (Malhado et al., 2013) and (El-Bramony, 2014). On the contrary to the present findings (Kawthar et al., 2005), (EL-Arian et al., 2012) and (Ibrahim et al., 2012) showed high heritability for days in milk and milk yield. Moderate heritability estimate was 0.18 and 0.19 for days open and calving interval, respectively. These results agreed with (Amin and Alhur, 2011) and (El-Bramony, 2014). The opposite results obtained by (Aziz et al., 2001), (Thevamanoharan et al., 2002), (Badran et al., 2005), (Kawthar et al., 2005), (El-Bramony, 2011), (Malhado et al., 2013) and (Barros et al., 2016) who recorded low heritability estimates for days open and calving interval.

Calving interval had high positive phenotypic correlation with days open (0.97) and dry period (0.88). Moreover, there were low positive

correlations with total milk yield (0.004). These results were in consistence with the findings obtained by (Aziz et al., 2001) and (Badran et al., 2005) showed high positive phenotypic correlation of calving interval with days open. Also, (Ramos et al., 2006) and (Malhado et al., 2013) recorded low positive correlation of calving interval with milk yield. There was high positive phenotypic correlation of days open with dry period (0.86). Moderate positive phenotypic correlation was found among days in milk and total milk yield (0.46). These results agreed with (Shalaby et al., 2013) reported that positive phenotypic correlation among days in milk and total milk yield. High positive genetic correlation was recorded between calving interval with each of days open (1.00), dry period (0.96) and days in milk (0.56). The obtained results were in the same line of those obtained by (Badran et al., 2005). On the contrary, opposite results obtained by (Aziz et al., 2001). There was high positive genetic correlation between days open with each of dry period (0.97) and days in milk (0.55) but low positive correlation with total milk yield (0.29) and 305day milk yield (0.25). These results disagreed with those reported by (Aziz et al., 2001). High positive genetic correlation between total milk yield with each of days in milk (0.73) and 305day milk yield (0.86). Similar to present results (Mourad and Khattab, 2009), (Ibrahim et al., 2012), (EL-Arian et al., 2012) and (Shalaby et al., 2013) recorded that high positive genetic correlation between days in milk and milk yield. The opposite results obtained by (Barros et al., 2016) reported that low negative correlation between days in milk and milk yield.

The breeding value for total milk yield and 305day milk yield of cows ranged between -380 and 370 and between -480 and 380 kg, respectively. Moreover, values for sires were between -310 and 240 and between -370 and 330 kg, respectively. While the corresponding values for dams were between -160 and 180 and between -230 and 170 kg, respectively. The opposite results obtained by (Cady et al., 1983) who found that expected breeding values for sires of Nili-Ravi Buffaloes for 250 to 305day milk yield averaged from -172 kg to +260, (EL-Arian et al., 2012), (Ismail, 2006), (Fooda et al., 2011) who showed that breeding value estimates for total milk yield was ranged between +101 to -269kg for Egyptian buffaloes.

There is one non synonymous SNP (A93G) at 93bp and led to change of methionine amino acid into isoleucine amino acid in exon 10 of FSHR gene (with 230bp size). The opposite results obtained by (Lan et al., 2006) showed that there was no mutation in exon 10 of FSHR gene with 230bp in Haimen goat. Also, there was no polymorphism of exon 10 of FSHR gene (with size 230 bp) detected in goat and this gene had only one genotype so this locus was homozygous by PCR-SSCP (Lei et al., 2010) and (Li et al., 2010). There was no polymorphism of exon 10 of FSHR gene (with size 306 bp) detected in Egyptian buffaloes which shown by only one pattern by PCR-SSCP. The obtained results were in the same line of those obtained by (Othman and Abdel-Samad, 2013) and (Sosa et al., 2015) showed that the PCR product for FSHR gene gave the specific band at size 306 bp and all examined buffaloes were genotyped as CC and there was none SNP in this gene by sequencing. The opposite results obtained by (Marson et al., 2008) observed that there were three genotype for FSHR gene (GG, CG and CC) in European-Zebu composite beef heifers from six different breed compositions by digestion with *AluI* restriction endonuclease Also, (Ahmed et al., 2011) who reported that there was polymorphism in FSHR gene locus with size 306bp by SSCP.

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