Original Research

Genetic Polymorphism of *CYP19* and *ER1* Genes and their Association with Cystic Ovarian Disease in HF Crossbred Cattle

H. Harini[1*], R. Nagaraja[2], S. Naveen Kumar[1], C. S. Nagaraja[3] and G. Sudha[4]

Veterinary College, Hebbal, Bengaluru, Karnataka, INDIA

[1]Department of Animal Genetics and Breeding
[2]Veterinary College, Gadag, Karnataka, India
[3]Department of Poultry Science
[4]Department of Veterinary Gynaecology and Obstetrics

***Corresponding author:** harinigowda822@gmail.com

Rec. Date:	Oct 30, 2019 12:30
Accept Date:	Nov 21, 2019 13:26
DOI	10.5455/ijlr.20191030123003

Abstract

The present study was carried out with the objective to determine the polymorphism of CYP19 (promoter) and ER1 genes and their possible association with cystic ovarian disease in HF crossbred cows. Promoter region of CYP19 and 5' Region of ER1 gene was amplified by PCR using published primers. The polymorphism in CYP19 and ER1 gene was detected using PCR-RFLP using PvuII and BglI restriction enzymes, respectively. PCR-RFLP detected three genotypes, AA (84%), AB (14%), and BB (2%) in HF crossbred cows for CYP9 gene and two genotypes GG and AG for ER1gene. The gene frequency of A and B alleles were 0.91 and 0.09, respectively for CYP19 gene and 0.12 and 0.88, respectively for A and G alleles for ER1 gene. The studied population was in Hardy Weinberg Equilibrium. The study revealed no association between CYP19/PvuII genotype and cystic ovarian disease in HF crossbred populations but 'AG' genotype of ER1 gene is associated with higher incidence of COD.

Key words: *CYP19*, Cystic Ovarian Disease, *ER1*, HF, Crossbred Cattle, PCR-RFLP, Polymorphism

How to cite: Harini, H., Nagaraja, R., Kumar, S., Nagaraja, C., & Sudha, G. (2019). Genetic Polymorphism of CYP19 and ER1 Genes and their Association with Cystic Ovarian Disease in HF Crossbred Cattle. International Journal of Livestock Research, 9(12), 212-220. doi: 10.5455/ijlr.20191030123003

Introduction

The reproduction and production traits are closely connected in cattle and therefore the genetic improvement of the reproduction traits is important for the efficient way of lactogenesis and lactopoesis. Generally, low numbers of genetic marker (AFLP, RFLP) significantly influencing the reproduction traits have been detected, because most of them are characterized by low heritability. One of the promising possibilities is the analysis of different genetic variants of hormones and protein factors mediating their

actions that have a key role in reproduction system (Szatkowska *et al*., 2011). Oestrogen biosynthesis is catalysed by the enzyme cytochrome P450 aromatase, which is encoded by *CYP19* gene. The cytochrome P450 aromatase is essential for physiology of reproduction (Conley and Hinshelwood, 2001). Othman *et al*. (2014) explored genetic polymorphism of exon 2 of *CYP19* gene and its association with ovarian activity in Egyptian buffaloes using PCR-SSCP and sequencing analysis. Substitution of nucleotide T by C at position 72 in the amplified fragments was related to the ovarian activity in Egyptian buffaloes. The aim of this study was to identify and characterize the genetic polymorphism in promoter region of *CYP19* gene by RFLP analysis and to elucidate the relationship between polymorphism in *CYP19* gene and COD in HF crossbred cattle. Numbers of molecular techniques together with conventional breeding methods are frequently making their impact in animal improvement in present era. Molecular techniques like detection of DNA-level polymorphism by restriction fragment length polymorphism (RFLP), AFLP, SNP and a number of molecular markers are in frequent use to improve animal performance from one generation to next generation (Khare & Khare, 2017).

In mammals, estrogens regulate many vital processes, such as reproduction, cell growth, differentiation, mammary gland development, lactogenesis, homeostasis and oncogenesis (Eng *et al*., 1997). Due to the numerous functions that estrogens play in the animals, estrogen receptors and their genes are considered candidates for the markers of production and functional traits in farm animals, including cattle. Estrogen exerts its effects through the estrogen receptors, a member of the nuclear steroid thyroid hormone receptors superfamily, which is expressed in a cell and tissue-specific manner. This specific pattern of estrogen receptors expression enables estrogens to direct their effects to target tissues (Green and Chambon, 1988). Estrogen and estrogen receptors (*ER*) play an important role in development and maintenance of the female reproductive system, maintaining fertility and the regulation of bone development and maintenance (Anderson *et al*., 1997).

Materials and Methods

The present study was undertaken on 155 Holstein Friesian (HF) crossbred cows from villages of Ramanagara District, adjoining villages of Bengaluru district and cows maintained at Department of Livestock Farm Complex (LFC), Veterinary College, Bengaluru, India. Study conducted for the period of two years between January, 2016 to December, 2017. The experimental animals were divided into two groups *viz*., COD affected and COD unaffected/ apparently healthy animals as control group. Identification of COD affected HF crossbred cows was done based on the history, clinical symptoms, per rectal palpation and ultrasound scanning of ovarian structures. The animals suffering from cystic ovarian follicle and the control animals both were taken for the study and the data regarding parity, body condition score was also recorded (Singh *et al.*, 2017). Genomic DNA was isolated from venous blood by following high salt method

 DOI 10.5455/ijlr.20191030123003

as described by Miller *et al.* (1988). Promoter region of *CYP19* gene was amplified by using published primer. The details of the primer and their expected product size are presented in Table 1. The primers were procured from Sigma Aldrich Chemical Pvt. Ltd., Bengaluru.

Table 1: Sequences of primers and expected sizes of PCR products of *CYP19 a*nd *ER1* genes

Genes	Primer sequence	Expected Product size (bp)	References
CYP 19 (promoter)	F: 5' CTCTCGATGAGACAGGCTCC 3'	405	Jedrzejczak *et al*., 2011
	R: 5' ACAATGCTGGGTTCTGGACT 3'		
ER1 (5' Region)	F : 5' TTTGGTTAACGAGGTGGAG	242	Szreder and Zwierzchowski, 2004
	R : 5' TGTGACACAGGTGGTTTTTC		

Each PCR reaction was done with a total volume of 25 µl consisting of a) 2x Red PCR Master Mix-12.5 µl (Tris-HCl pH 8.5, $(NH4)_2S0_4$, 3 mM MgCl2, 0.2% Tween 20, 0.4 mM of each dNTP, 0.2 units/µl Ampliqon Taq DNA polymerase, Inert red dye and stabilizer), b) Forward primer - 1 µl (2 pmol/µl), c) Reverse primer - 1 µl (2 pmol/µl), d) DNA template -1 µl (50-100 ng), e) Nuclease free water - 9.5 µl. The cycle conditions included an initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 15 sec, annealing at 59 °C for 15 sec and extension at 72°C for 2 min and final extension at 72°C for 5 min for *CYP19* gene. The cycle conditions included an initial denaturation at 94 °C for 1 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 1d0 min for *ER1* gene. The amplified PCR products of the *CYP19* and *ER1*gene was resolved on 1.5 per cent agarose gel with 100 bp DNA ladder at a constant voltage of 100 V for 45 to 60 min. The gel was visualized under a Gel documentation system (Biorad Molecular imager Gel Doc XR+, USA). The RE digested product of *CYP19* (promoter) and 5' Region of *ER1* gene was resolved on 2.0 per cent agarose gel with 100 bp ladder at a constant voltage of 100 V for 90 min. The details of RE used and recognition site are presented in Table 2.

Table 2: Restriction enzymes used for digestion of PCR products of *CYP19* and *ER1* genes

S. No	Gene	Restriction Enzyme	Recognition Site
3	*CYP19*	*Pvu*II	5'…CAG ↓CTG …3′
			3′… GTC GAC …5′
5	*ER1*	*Bgl*I	5'... GCCNNNN↓NGGC...3'
			3'...CGGNNNNNCCG...5'

The resultant patterns of electrophorosed DNA was photographed and analyzed using gel documentation system. PCR products of representative samples of resultant patterns were sent for sequencing at Eurofins Genomics India Pvt. Ltd., Bengaluru. The sequences obtained were analyzed, consensus was created, annotated and multiple sequence analysis was performed by using CLC Main Work Bench Software (CLC

 DOI 10.5455/ijlr.20191030123003

BIO 2011, USA). All statistical analyses for determination of associations between *CYP19* and *ER1*genotypes and COD in Holstein Friesian crossbred cows were performed by Chi-square test using GraphPad prism software (GraphPad prism version 6.05).

Results and Discussion

Identification of COD affected HF crossbred cows was done based on the history, clinical symptoms, per rectal palpation and ultrasound scanning of ovarian structures.

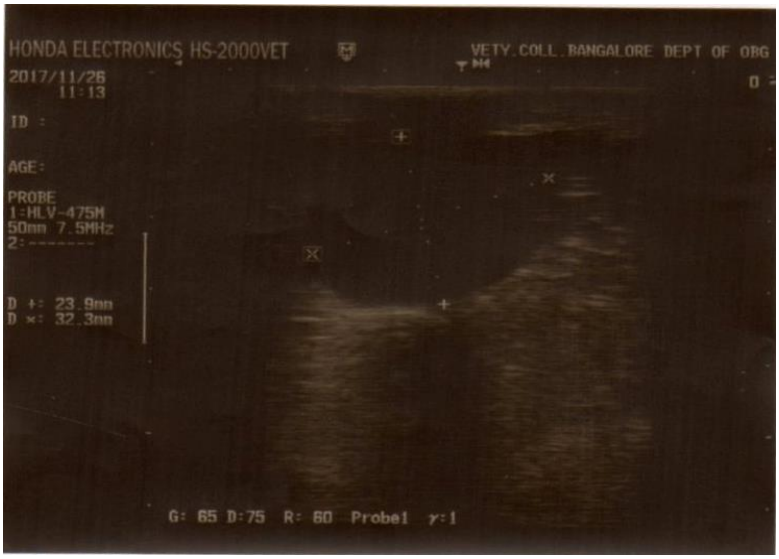

Fig. 1: Ultrasonograph of cyst showing diameter of 23.9X 32.3 mm

The amplified PCR products were resolved on 1.5 per cent agarose gel. The size of the amplified products for *CYP19* (promoter) gene and *ER1* (5' region) gene in the studied population was 405 bp and 248 bp, respectively (Fig. 2 & Fig. 3).

DOI 10.5455/ijlr.20191030123003

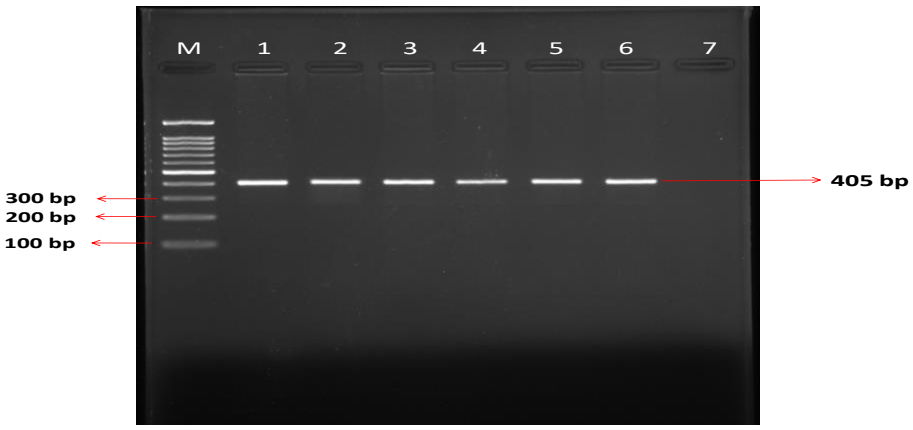

Fig. 2: Agarose gel (1.5 %) showing PCR amplified product of *CYP 19* (promoter) gene. Lane M: Molecular marker (100 bp DNA ladder), Lanes 1, 2, 3, 4, 5, 6: PCR amplified product 405 bp (HF crossbred), Lane 7: No Template Control.

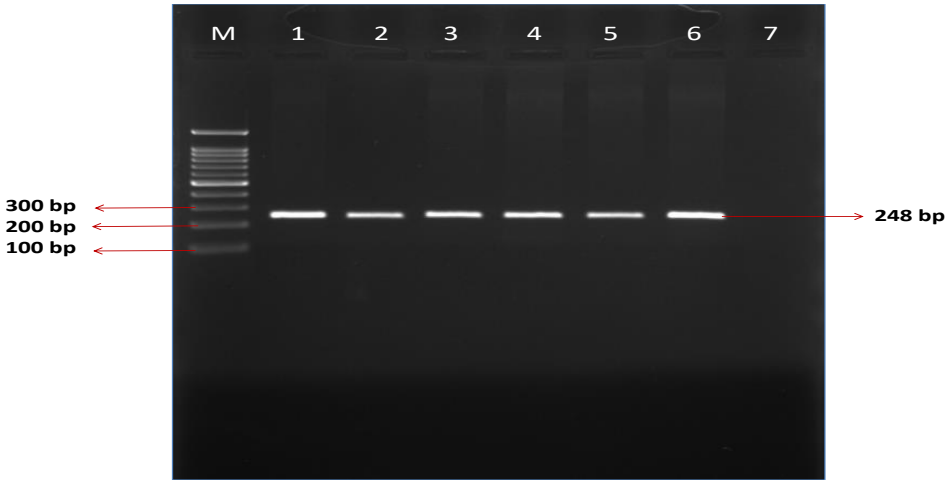

Fig. 3: Agarose gel (1.5 %) showing PCR amplified product of *ER1* (5' region) gene. Lane M: Molecular marker (100 bp DNA ladder), Lanes 1, 2, 3, 4, 5, 6: PCR amplified product 248 bp (HF crossbred), Lane 7: No Template Control.

Digestion of PCR amplicons of *CYP19* (promoter) gene by *Pvu*II restriction enzyme exhibited three fragments of 78, 327 and 405 bp, on 2.0 per cent agarose gel electrophoresis, resolving into three genotypes in HF crossbred cows. The three genotypes were AA represented by 405 bp fragments, BB represented by 327 and 78 bp fragments and AB represented by 405, 327 and 78 bp fragments (Fig. 4). All three genotypes of *CYP19/PvuII* were detected and analyzed in population of Holstein Friesian crossbred cows. The *CYP19* variants were studied in 155 animals of HF Crossbred cows, out of which, 130 were AA genotype, 22 animals were of AB genotype and 03 animals were BB genotype. The genotypic frequency was 0.84, 0.14 and 0.02 for AA, AB and BB, respectively. Among three genotypes in CYP19 frequency of AA was highest, which is in agreement with the reports of Szatkowska *et al*. (2011) and Trakovicka *et al*. (2015) in Polish Holstein cows and Slovak Simmental cows, respectively. The allelic frequencies in HF crossbred cows

DOI 10.5455/ijlr.20191030123003

were 0.91 and 0.09 for A and B, respectively. The frequency of A allele was higher, and is in agreement with the findings of Szatkowska *et al*. (2011) and Jedrzejezak *et al*. (2011) in Polish Holstein cows and Black and White cows, respectively.

In the present study, the amplified product of *ER1* gene was digested with *Bgl*I restriction enzyme which revealed two different allelic patterns in HF crossbred cows, on 2.0 per cent agarose gel electrophoresis. The first allelic pattern showed two bands of size 171 and 77 bp and was denoted as GG genotype. Second allelic pattern showed three fragments of size 248, 171 and 77 bp, which was classified as AG genotype (Fig. 5). In HF crossbred cows, absence of AA genotype was evident. In case of *ER1* genotypes. These results are in agreement with the earlier reports of Othman (2013) in Egyptian buffalo and Zaborski and Grzesiak (2011) in Holstein Friesian and Keskin *et al.* (2015) in HF subfertile heifers. Contrarily, Jedrzejezak *et al.* (2011), Szatkowska *et al.* (2011) and Keskin *et al.* (2015) have reported the presence of three genotypes (AA, AG, GG) in Black and White, HF cows and fertile HF heifers, respectively.

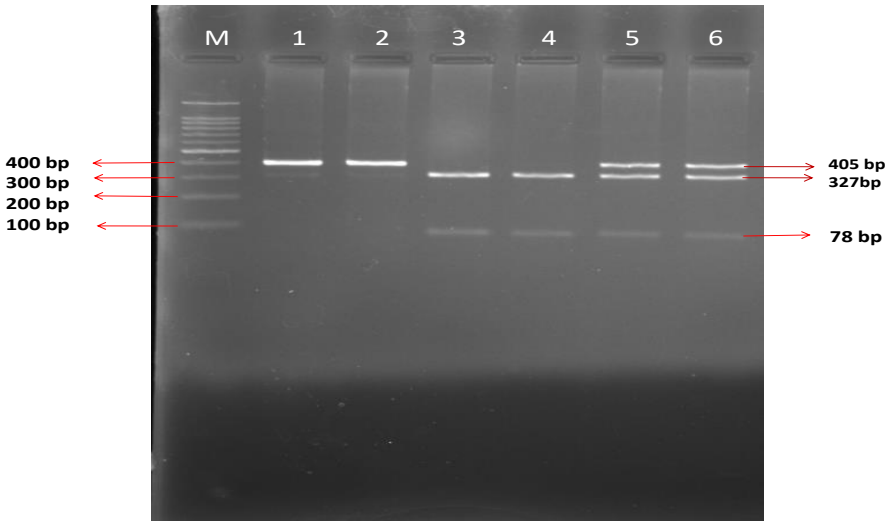

Fig. 4: Agarose gel (2.0%) showing PCR product after digested with restriction enzyme *Pvu*II. Lane M: Molecular Marker (100 bp DNA ladder), Lane 1 & 2: Homozygous genotype AA in HF crossbred (405 bp), Lane 3 & 4: Homozygous genotype BB in HF crossbred (327 and 78 bp), Lane 5 & 6: Heterozygous genotype AG in HF crossbred (405, 327 & 78 bp).

DOI 10.5455/ijlr.20191030123003

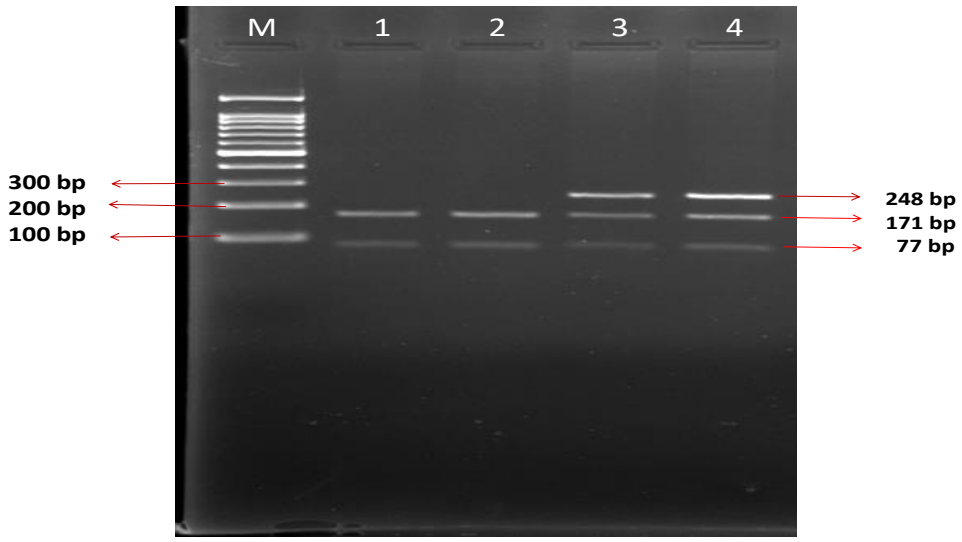

Fig. 5: Agarose gel (2.0 %) showing PCR product after digested with restriction enzyme *Bgl* I for detection of *ER1* (5' region) gene polymorphism in HF crossbred cows. Lane M: Molecular Marker (100bp DNA ladder), Lane 1 & 2: Homozygous genotype GG in HF crossbred (171 and 77 bp), Lane 3 & 4: Heterozygous genotype AG in HF crossbred (248, 171 & 77 bp).

Further, the observed and expected heterozygosities were 0.1419 and 0.1640 in HF crossbred cows. The $\chi 2$ test showed that the studied population was in Hardy–Weinberg equilibrium. The BLAST search of sequence of *CYP19* gene for possible match yielded around 10 hits in the NCBI nucleotide data base. Among these 100 percent identity was observed with accession number KT596709.1 of *Bos taurus.* Alignment of A and B allele sequences of *CYP19* gene using CLC Main Work bench 6.8.1, showed a SNP A>G transition at position 78 in restriction site and it was confirmed on chromatogram (Fig. 6). Chi-square analysis of genotypic frequencies in HF crossbred cows revealed the observed and expected heterozygosities of 0.232 and 0.205, respectively, and the studied HF crossbred population was in Hardy Weinberg equilibrium with respect to studied *ER1* locus. The BLAST search of annotated sequence of *ER1* gene for possible match yielded around 65 hits in the NCBI nucleotide data base. Among these 100 per cent identity was observed with accession number AY332655.1 of *Bos taurus*.

DOI 10.5455/ijlr.20191030123003

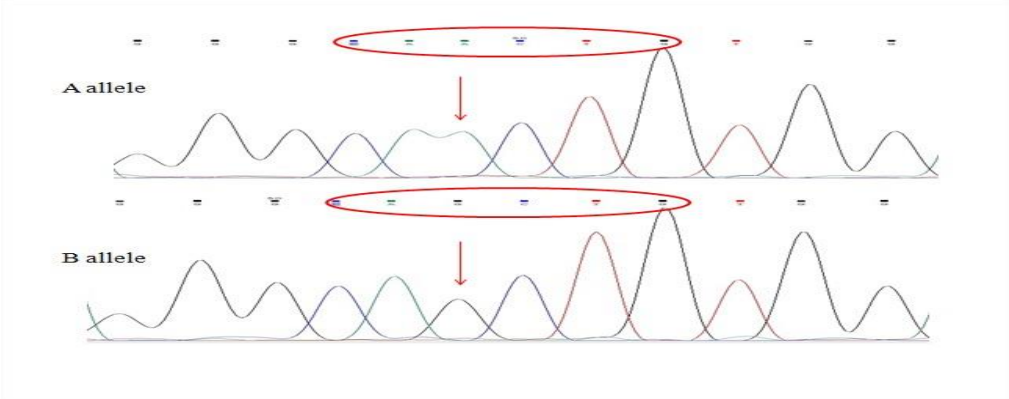

Fig. 6: Chromatogram showing A>G transition for CYP19 (promoter) gene at position 78 of the PCR amplified product (restriction site)

The present study revealed no significant association of *CYP19* genotypes with COD in HF crossbred cows. Contrary to results of present study, Polish HF cows with CYP19AA genotypes were found to have longer Calving to Conception interval (CLVCs) compared to heterozygotes and this difference was significant in first and third lactation (P<0.05) Similarly, the average Calving interval (CLVIs) were longer in CYP19AA homozygotes than in heterozygous cows; however, significance was proven only in the third lactation (Szatkowska *et al*., 2011. Further, Trakovicka *et al*. (2015) have reported the significant effect of *CYP19*/ *Pvu*II genotypes on Milk Yield (MY) and Protein Yield (PY). They have reported the higher production of milk yield and protein yield in individuals with BB genotypes.

Present analysis revealed the significant association (P<0.05) of AG genotype of *ER1* with the incidence of COD in HF crossbred cows. The incidence of COD was highest in HF crossbred cows with AG genotype of *ER1* gene. Similarly, genotypes of *ER1* gene were found to significantly influence other reproductive traits. Significantly shorter CLVC ((P<0.05) was observed in cows of *ER1*/ *Bgl*I GG genotype compared to heterozygotes (AG) in the 1st lactation. Similarly, longer calving to conception interval and longer calving interval were reported in *ER1* homozygous cows compared to heterozygotes in the 3rd lactation (Szatkowska *et al*., 2011).

Conclusion

Substantial evidence existence for presence of genetic variability in *CYP19* and *ER1* genes in HF crossbred cows. In HF crossbred populations, 'AG' genotype of *ER1* gene is associated with higher incidence of COD. *ER1* genes in HF crossbred cows may be considered as candidate genes for selection of COD risk free animals, but suitable validation and confirmation in larger populations is necessary. *CYP19* gene variants showed no association with COD in HF crossbred cattle population studied. However, these results warrants further robust studies to arrive at definitive conclusions. "Dissemination Scenarios" must be developed to extend the genetic progress from lab to land.

 DOI 10.5455/ijlr.20191030123003

Acknowledgments

The authors are thankful to Karnataka Veterinary Animal and Fisheries Sciences University for providing the funds to carry out the work.

Conflict of Interest

None of the authors of this paper have a financial or personal relationship with other people or organization that could inappropriately influence or bias the content of the paper.

References

1. Anderson, T. I., Wooster, R., Laake, K., Collins, N., Warren, W., Skrede, M., Elles, R., Tveit, K. M., Johnston, S. R., Dowsett, M., Olsen, A. O., Moller, P., Stratton, M. R. and Dale, A. L. 1997. Screening for ESR mutations in breast and ovarian cancer patients. *Hum Mutat*., 9: 531-536.
2. Conley, A. and Hinshelwood, M. 2001. Mammalian aromatases. *Reproduction*, 121: 685-695. Eng, F. C. S., Lee, H. S., Ferrara, J., Willson, T. M. and White, J. H. 1997. Probing the structure and function of the estrogen receptor ligand binding domain by analysis of mutants with altered transactivation characteristics. *Mol. Cell. Biol*., 17: 4644- 4653.
3. Graphpad PRISM VERSION 6.05 FOR WINDOWS, GraphPad software, San Diego California USA, www.graphpad.com"
4. Green, S. and Chambon, P. 1988. Nuclear receptors enhance our understanding of transcriptional regulation. *Trends Genet*., 4: 309–314.
5. Jędrzejczak, M., Grzesiak, W., Szatkowska, I., Dybus, A., Muszyńska, M. and ZaborskI, D. 2011. Association between polymorphisms of *CYP19*, *CYP21*, and *ER1* genes and milk production traits in Black-and-White cattle. *Turk. J. Vet. Anim. Sci.*, 35(1): 41-49.
6. Keskin, A., Oner, Y., Yilmazbaş-Mecitoglu, G., Guner, B., Karakaya, E., ElmacI, C. and Gumen, A. 2015. Distributions of CYP19, ERα and PGR Allele Frequencies between fertile and subfertile Holstein-Friesian heifers. *Journal of the Faculty of Veterinary Medicine*, 21(6): 893-898.
7. Khare, V., and Khare, A. 2017. Modern Approach in Animal Breeding by Use of Advanced Molecular Genetic Techniques. *International Journal of Livestock Research*, 7(5), 1-22.
8. Miller, S. A., Dykes, D. D. and Polesky, H. F. 1988. A sample salting out procedure for extraction of DNA from human nucleated cells. *Nucleic Acids Res*., 16: 1215.
9. Othman, O. E., Ahmed, W. M., Balabel, E. A., Zaabal, M. M., EL Khadrawy, H. H. and Hanafy, E. M. 2014. Genetic Polymorphism of *Cyp19* Gene and its association with ovarian activity in Egyptian Buffaloes. *Global Veterinaria*, 12(6): 768-773.
10. Singh, M., Honparkhe, M., Kumar, A., Ghuman, S., and Singhal, S. 2017. Comparative Efficacy of Cystic Ovarian Follicle Ablation and CIDR Based Ovsynch in Dairy Cattle. *International Journal of Livestock Research*, 7(5), 175-182.
11. Szatkowska, I., Grzesiak, W., Jędrzejczak, M., Dybus, A., Zaborski, A. and Jankowiak. D. 2011. An analysis of CYP19, CYP21 and ER genotypes in Polish Holstein-Friesian cows with regard to the selected reproductive traits. *Acta Vet. Brno.*, 80: 65–71.
12. Szreder, T. and Zwierzchowski, L. 2004. Polymorphism within the bovine estrogen receptor-α gene 5'-region. *J. Appl. Genet*., 45: 225-236.
13. Trakovická, A., Moravčíková, N., Miluchová, M. and Gábor, M. 2015. Analysis of cyp19 gene polymorphism as factor affecting milk production of cattle. *Journal of Microbiology, Biotechnology and Food Sciences*, 4(2): 111-113.
14. Zaborski, D. and Grzesiak, W. 2011. Detection of heifers with dystocia using artificial neural networks with regard to ERα-BglI, ERα-SnaBI and CYP19-PvuII genotypes. *Acta Sci. Pol., Zootechnica.*, 10 (2): 105–116.

 DOI 10.5455/ijlr.20191030123003