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# Genomic Study of Silent Heat: A Novel Mutation in *HSD17β1* Gene Muting the Potential of Black Gold of Asia

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## ABSTRACT

Buffaloes are useful in the conversion of straw into agro-industry waste and bio-fertilizer, in addition to giving milk, meat, and mechanical power to humans. Between 65 and 60% of the world's milk is produced by buffaloes. Regardless of their excellent production capabilities, buffalo production is challenged due to poor fertility, delayed maturity, poor estrus behavior and seasonality in breeding. Buffaloes have a high level of genetic variation, which might affect the buffalo estrus cyclicity. When female animals exhibit estrus behavior, male animals are attracted to their female counterparts, and this is known as the mating instinct. Buffalo being a shy breeder depicts the estrus behavior late night. Very hot climate may also affect the estrus behavior. But in majority of the cases estrus signs are not visible enough to ensure the mating. Various genomic regions have been identified affecting poor estrus behavior in buffaloes. In the current study, novel insertion (p.3491) was found in HSD17β1 gene which is a key role player in production of estradiol. Polymorphic site was identified by comparing the genomic regions of animals with higher and lower heat score (≥50). This polymorphism was further analyzed for Hardy Weinberg Equilibrium (HWE), allelic frequency, heterozygosity status and association with heat score calculated by considering exhibited heat signs by animals. A chi-square value of 2.08 ($P > 0.05$) depicted that genotypic distribution of locus was obeying HWE in the population under study. Allelic frequency of the mutant allele was lower (0.39) but association testing for the genotypic distribution provided significant values (17.47±0.25). Three dimensional protein structure illustrated the variation between residues 150 to 180. Truncation of five residues was also observed in the length of the mutant protein. Protein network was also constructed to understand the interaction of protein. Functional significance of the identified polymorphic sites illustrates the use of these regions as targeted site for marker assisted selection or genome editing, which would enhance the reproduction potential of river buffalo.

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**Key words**
**Silent estrus, *HSD17β1* gene, River buffalo, Polymorphism**



## INTRODUCTION

Agriculture contributes 50 percent to the national economy of Pakistan, while livestock contributes 14.0 percent in overall GDP (Pakistan Economic Survey, 2022-23). Among major livestock species, buffalo stands out as well adaptive unique animal of temperate regions. In addition to providing humans with milk, meat, and mechanical power, buffaloes are also beneficial in converting straw into agroindustrial waste and bio-fertilizer. Buffaloes provide 65-60% of the world's milk (Riaz *et al*., 2018). Asia accounted for 97.10 percent of the world's buffalo milk production in 2007, which totaled 86,574.5 thousand tons (Pasha and Hayat, 2012). Due to their remarkable productive capabilities, these are denoted as black gold of Asia. Success of buffalo production is highly dependent upon the high fertility and regularity of estrus cyclicity of the animal (Siddiky and Faruque, 2017). Due to weak expression of estrus symptoms in buffaloes, a varied estrus cycle, delayed and low conception rate and repeated act of breeding have become particularly challenging (Sartori and Barros, 2011). This is an area where improvements can be made to increase reproductive efficiency (Pasha and Hayat, 2012; Kumar *et al*., 2013).

Detecting a buffalo throughout its reproductive cycle is extremely challenging because of its homosexual

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tendencies (Suthar and Dhami, 2010). Bison exhibit estrus behavior from September to January as a result of seasonal influences. October and November are the months when this habit peaks (Kumar *et al*., 2013). Because the river buffalo does not display signs of heat from March to June, it is assumed that the buffalo's reproductive season lasts eight months, and that it is sexually dormant for four months (Suthar and Dhami, 2010; Phogat *et al*., 2016). Hormones such as testosterone are formed by 17b-hydroxysteroid dehydrogenases (17b-HSDs). Scientists have studied seven types of HSDs used in sex hormone production and inactivation (Lu *et al*., 2020). Low levels of ovarian estrogens are the primary reason of buffalo's infertility, which causes anestrus stage. Estrogen production depends on cytochrome P450 aromatase, which is represented by CYP19 (Stevenson *et al*., 2014; Imran *et al*., 2018). Hormones such cytochrome P450 aromatase (CP450) and HSD17β1 are synthesized with estrogen (Kumar *et al*., 2009).

Oestradiol-17 is found in high concentration in medium-sized ovarian follicles, compared to bigger and smaller follicles (Verma *et al*., 2014). Climatic conditions such as hot-dry or hot-humid weather affect its concentration as well. As the temperature rises, the concentration of estradiol drops. A lack of estrus heat causes buffaloes to be hesitant breeders. Silent estrus behavior is affected by multiple genetic and environmental variables. *HSD17β1* gene is thought to be a crucial player in the synthesis of 17-b estradiol (Liu *et al*., 2009). In the given context, study was planned to identify polymorphisms in *HSD17β1* gene associated with silent estrus in the Nili-Ravi buffalo breed. Animals were selected from the animal farms at University of Veterinary and Animal Sciences, Ravi campus, Pattoki (n=192) and exonic regions of the gene was amplified and sequenced. SNPs were identified by comparing the sequences obtained and then analyzed for the statistical significance. Protein configuration was also predicted to see the functional consequences of the polymorphic sites on the protein. Identified genomic polymorphic sites affect the estrus behavior in buffaloes and can be used as candidate sites for the gene-based therapeutic approaches to combat the issue of silent estrus.

## MATERIALS AND METHODS

*Taxonomic species*

*Bubalus bubalis* was the species employed in this investigation. Nili Ravi buffalo breed of Pakistan was selected. River buffalo is well adapted in the hot climatic conditions of the Punjab province in Pakistan. Sampling was conducted from the Animal Farm at University of Veterinary and Animal Sciences, Ravi campus, Pattoki, Pakistan. Animals with typical phenotypic features for the Nili Ravi buffalo breed were selected for the study.

*Sampling strategy*

Nili Ravi buffaloes were tracked for their estrus cyclicity for consecutive three months and estrus record was used to calculate the heat score of animals by using the score chart reported by Van Eerdenburg *et al*. (1996). Animals with estrus score more than 50 were put into control group (n=93) and animals with estrus score less than this were put into test population (n=99). Along with the reproductive history, production records of the animals were also observed. Animals with disease history or no previous tract for parturition were excluded from the study. Blood sampling of the selected animals (n=192) was conducted between August-December, 2019 as these months are considered as breeding season. Blood was collected by pricking the jugular vein and stored into EDTA coated vacutainers. Samples were immediately moved to the Molecular Lab in Institute of Biochemistry and Biotechnology for further process and kept at -20°C prior to DNA extraction.

*Genomic DNA extraction and quantification*

Organic method of genomic DNA isolation was used as reported by Sambrook and Russell, 2001. Phenol-chloroform-isoamyl alcohol was used to precipitate the protein contaminants from the cell residues and DNA was obtained. Quantification of the genomic DNA was done by using Nanodrop Spectrophotometer at A260/A280 and sample concentrations were standardized at 50 ng/ml.

*Genetic characterization of* HSD17β1 *gene*

Primer designing and synthesis: *HSD17β1* gene is located on chromosome 3 of the bovines and carries six exons. Primer 3 (https://primer3.ut.ee/) was used to design the gene's specific primer pairs (Table I). Oligocalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) was used to evaluate the hairpin formation, self-annealing, and similarities and other secondary structural components. Suitably designed primer sets were synthesized by commercial facility.

Primer amplification and sequencing of amplicons: Primers were optimized for the optimal amplification according to the varying annealing $T_m$ ranges. Amplification was done by conventional polymerase chain reaction using the template, dNTPs, Taq polymerase, bi-valent ions and buffer in the master mixture. Primers were added at concentration of 10pM. Amplicons were purified using QIAquick PCR Purification Kit (Cat. No: 28104). Purified products were then sequenced bidirectionally (Sanger's chain termination method) via commercial facility.

**Table I. Primer pairs for *HSD17β1* gene in river buffalo.**

| Primers | Sequence 5`→3` |
|---|---|
| Exon 1 | F TGGAGAACTCCATGGACAGA |
| | R TACTTAGGCCGGGAAGGAGT |
| Exon 2 | F TAAGGCTGAAAGACCGGAAT |
| | R GTGGGCTTCTGGACATCTTG |
| Exon 3/4 | F AACAAGCCCAAGAGCCTTCT |
| | R AGAGCGGTTCCAACAAGATG |
| Exon 5 | F GCTGTAAACCCGCTTTCAAAT |
| | R ATAGCGCCTGGAGGAAGAC |
| Exon 6 | F AGGAGGTGGTCGAGGTAAGC |
| | R GCGGATTAGGCCTTTATTGC |

*Bioinformatics and statistical analysis*

Sequences obtained were analyzed by sequence alignment software (https://www.genome.jp/tools-bin/clustalw) and genomic variations were identified in the test and control animal groups. Identified loci were further analyzed for Hardy-Weinberg equilibrium (HWE) by chi-square testing and allelic and genotypic frequencies were also calculated by using POPGENE32 (https://sites.ualberta.ca/~fyeh/popgene.pdf). Genomic loci obeying the HWE were further analyzed for association testing by VassarStats (http://vassarstats.net/).

*3D protein structural configuration*

3D protein model was predicted for the standard and mutated polypeptides by using Phyre2 Protein Fold Recognition Server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). Secondary structural elements as transmembrane loop and domains were also predicted. Interaction of HSD17β1 protein was determined by using String (https://string-db.org/).

## RESULTS

*Identification of SNP in HSD17β1*

In this study, we analyzed the data of 192 Nili-Ravi buffaloes categorized in control (n=93) and test population (n=99). Sequencing of exonic regions of *HSD17β1* gene illustrated an insertion of "G" base at position 3491 in the gene (*XM_006055742.2*). Insertion was identified after sequence alignment of *Bubalus bubalis* with sequences obtained from the Nili-Ravi buffaloes of Pakistan. Identified polymorphic site was further analyzed for the amino acid substitution by ExPaSy Protein Translation Tool and substitution of all subsequent amino acids in the exon-2 was observed (Fig. 1).

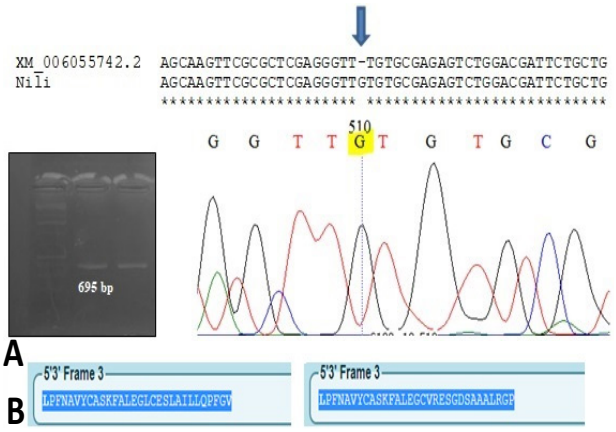
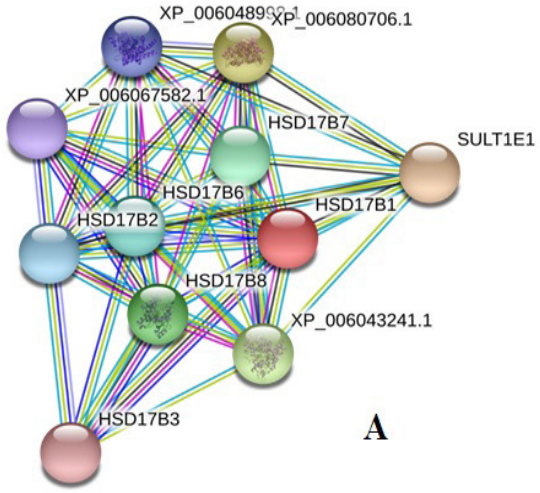



Fig. 1. Base insertion (G) in Exon-2 of *HSD17β1* gene in Nili-Ravi buffalo. A, Sequence alignment of *Bubalus bubalis* (XM_006055742.2) with Nili-Ravi buffalo of Pakistan indicated the insertion of 'G' base at position 3491. B, p.3491 indicated the shifting of amino acid sequence of subsequent chain.

*HWE and association analysis of the p.3491*

Identified locus (p.3491) of *HSD17β1* gene in Exon2 (Mutant G with frame shift mutation) in Nili-Ravi buffalo was further analyzed for HWE to observe the effect of mutation in population statistics. A chi-square test was performed yielding a value of 2.08 with $P > 0.05$. As locus was non-significant, so it was fixed in the population's gene pool structure by obeying the HWE law. The allelic frequency of the locus was also calculated (A, 0.60; B, 0.39). Shannon index is the measure of locus diversity and richness. Its value for the locus was 0.67 which is medium to high score depicting locus is diversed (na=2, ne=1.91). Summary of heterozygosity for the locus was observed as 0.2857, which was lower than the expected value 0.48857 (Table I). This depicts the increased ratio of homozygous loci among studied population. These results support our assumption that genotypic distribution of locus was obeying HWE in the population under study.

Results of association analysis suggested that insertion p.3491 was significantly associated with the lowered heat score (<50) of the animals during the estrus cycle (Table II). Mutant genotype scores were observed as (17.47±0.25), while heat score of the wild genotypes was much higher (53.95±0.52). These values were significantly associated with heat scoring in the bovines (P-value>0.05). Allelic frequency of the mutant form was lower (0.39) but it imparts a strong effect on the estrus behavior of the river buffalo.




| Protein | Details |
|---|---|
| *SULT1E1* | *Derived by automated computational analysis by using gene prediction method-Gnomon.* |
| *XP-006080706.1* | *Cytochrome P450 1A1 like isoform X1 (516 aa).* |
| *XP-006043241.1* | *Cytochrome P450 19A1 like (401 aa)* |
| *HSD17B8* | *Estradiol 17-beta-dehydrogenase 8 isoform X1 (260 aa)* |
| *HSD17B7* | *Estradiol 17-beta-dehydrogenase 7. Derived by automated computational analysis by using gene prediction method-Gnomon.* |
| *HSD17B6* | *Estradiol 17-beta-dehydrogenase 6. Derived by automated computational analysis by using gene prediction method-Gnomon.* |
| *HSD17B3* | *Estradiol 17-beta-dehydrogenase 3 (388aa)* |
| *HSD17B2* | *Estradiol 17-beta-dehydrogenase 2. Derived by automated computational analysis by using gene prediction method-Gnomon.* |
| *XP-006067582.1* | *Estradiol 17-beta-dehydrogenase 12 B like (349aa)* |
| *XP-006043998.1* | *Derived by automated computational analysis by using gene prediction method-Gnomon.* |

B

Fig. 2. Prediction of functional protein association network of *HSD17β1* protein in *Bubalus bubalis*. A, HSD17B1 indicated a complex functional linkage with other members of estradiol 17 beta dehydrogenases. B, shows the details of abbreviations.

**Table II. Heterozygosity statistics for p.3491 in HSD17β1 in Nili-Ravi buffalo (Mean±SD).**

| Locus | Obs_ Hom | Obs_ Het | Exp_ Hom* | Exp_ Het* | Nei** | Ave_ Het |
|---|---|---|---|---|---|---|
| p.3491 | 0.714± 0.00 | 0.285± 0.00 | 0.514± 0.00 | 0.485± 0.00 | 0.477± 0.00 | 0.477± 0.00 |

*Expected homozygosity and heterozygosity were computed using Levene (1949). **Nei's (1973) expected heterozygosity.

**Table III. Association analysis of p.3491 with heat scores (Van Eerdenburg *at al.* 1996) by one way ANOVA using Vassar stats (Mean±SEM).**

| SNPs | Association analysis (n=192) | | | Probability (P < 0.05) |
|---|---|---|---|---|
| | AA (n=70) | AB (n=12) | BB (n=110) | |
| p.3491 | 53.95±0.52 | 42.9±0.13 | 17.47±0.25 | <.0001 |

*3-D protein configuration of* HSD17β1

Protein association network was also predicted and a complex functional linkage of the protein was observed suggesting its involvement in numerous essential pathways (Fig. 2). Protein structure prediction of the HSD17β1 illustrated the change in protein folds between residues 150 to 180. Truncation of five residues was also observed in the length of the mutant protein (Fig. 3A). A transmembrane helix was identified between 113-128 residues (Fig. 3B). Secondary structural entities were also identified depicting the distribution of helices and coils (Fig. 3C).

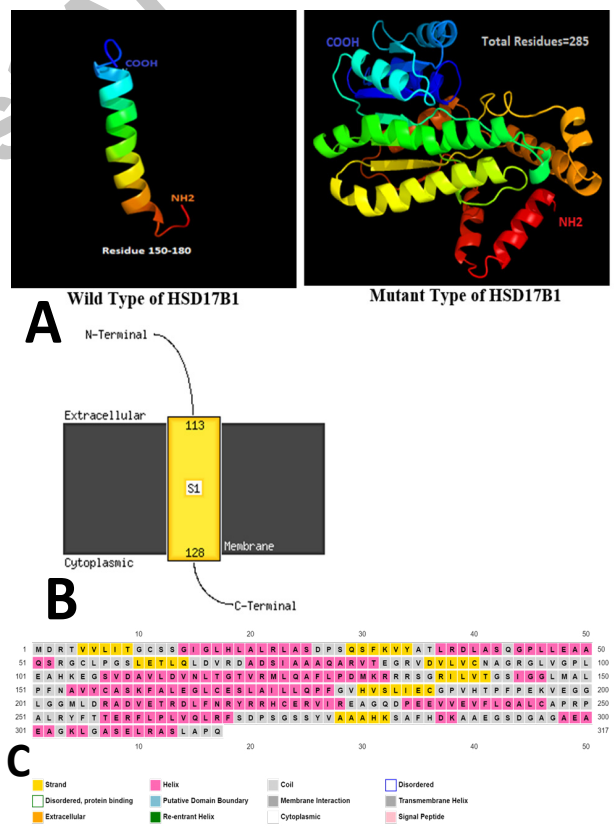



Fig. 3. HSD17β1 protein. A, 3-D protein model prediction by fold recognition method; B, trans-membrane helix between 113 to 128 residues; C, secondary structure prediction by PSIPRED.

## DISCUSSION AND CONCLUSION

River buffalo is mostly referred as black gold due to its superior production capabilities. But poor reproductive traits stand as a hindrance in exploring the potential of this animal (Perera, 2011). 17b-HSD is involved in the production of 17-b estradiol and is encoded by the *HSD17β1* gene. In the ovaries, this enzyme is synthesized and exhibits lipid-like characteristics. Female mammals exhibit estrus activity under the effect of 17-b estradiol. Estradiol is a hormone that acts on the brain to elicit bodily estrus sighs. As the uterus prepares for pregnancy, this hormone increases the blood flow to it. Its concentration rises during the estrous phase of the estrus cycle. A huge buffalo population has a very low concentration, which causes it to develop quiet heat symptoms (Kommadath *et al*., 2011). Inadequate nutrition, poor management, infections, hereditary causes, genomics and other environmental variables are among the causes of the epidemics. This information can be used to forecast whether a buffalo will exhibit silent estrus or not.

Buffalo's silent heat is caused by a surge of gonadotropins just before and during the pre-ovulatory surge of estradiol. It was first published in (Bachalaus *et al*., 1979). This occurs because the concentration of progesterone and estradiol decreases during the estrus cycle. Because of this, progesterone concentrations reduce the peak values of estradiol around estrus (Rao *et al*., 1982). This enzyme, also known as 17beta-HSD, is responsible for the final step in estrogen production. Esterification is the principal activity of HSD17β1, which catalyzes the final step of estradiol production (Babitha *et al*., 2013; Yazawa *et al*., 2023). 17-HSD 17-HSD1 is the most abundant enzyme in the ovaries, and it is engaged in the final phase of 17-b estradiol metabolism in the ovaries by follicles, according to the National Institutes of Health (Mitko *et al*., 2008).

In this study exonic regions of *HSD17β1* gene were analyzed to identify novel variations affecting the estrus behavior in river buffalo. Nili-Ravi Buffalo animals from Punjab province of Pakistan were included in the study. On the basis of heat scoring, animals were categorized into control (>50) and test population (<50). Genome sequencing provided with the insertional mutation (p.3491) at position 3491 in exon-2 of the gene affecting the subsequent amino acid chain. Bioinformatics and statistical analysis of the locus and 3-D protein configuration analysis of the protein illustrated the significant effect of the insertion on estrus behavior of the population.

In past, it was believed that season plays a significant role in the regulation of estrus but now the influence of structure and regulation pattern of various genes has been reported in numerous studies effecting the poor manifestation of estrus behavior (Barkawi *et al*., 2009; Boer *et al*., 2010; Mirmahmoudi and Parkash, 2012; Zobel *et al*., 2012; Jia *et al*., 2015). This results in laborious and frequent tracking of animal's reproductive tract to find the suitable time for insemination. Missing one cycle not only delays the animal's reproductive cycle but also affects the farmers. Genomic association of this trait provides a promising insight into candidate genes. Suitable approaches for early age detection and cure can be developed to combat the issue of silent estrus in river buffalo.



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*Ethical approval*

Research work was conducted with due approval of the Institutional Research Ethical Committee.

*Consent to participate*

All authors of the manuscript are agreed upon the publication in the submitted form.

*Consent for publication*

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*Statement of conflict of interest*

Ther authors have declared no conflict of interest.

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