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Pretentious genomic selection signatures in *CYP19A1* gene associated with silent estrous behavior in water buffalo in Pakistan

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ABSTRACT

Background: Poor reproductive efficiency of river buffalos hampers the production capabilities of animals. Buffalos are mainly considered poor breeders owing to the constrained expression of estrus behavior. Failure to display heat signs is an indication of improper functionality of signaling peptides to trigger on a series of behavioral changes, which can be detectable by breeders for timely insemination of females. This might cause an animal to be a repeat breeder. Genomic variations underlying synthesis of signaling peptides can be a useful marker to select superior animals with better reproductive efficiency. In this context, the current study was designed to analyze the *CYP19A1* gene in Nili-Ravi buffalo.
Results: A total of 97 animals were selected and were divided into two groups on the basis of their heat score. PCR amplification and sequencing of the amplicons were performed using the specific sets of primer, and then, sequences were analyzed for novel variants. A total of 11 polymorphic sites were identified illustrating phenotypic variation in the heat score. Most of the loci were found homologous. Single Nucleotide Polymorphisms (SNPs) were analyzed for association with silent estrus. A three-dimensional protein model was also generated to locate the position of exonic SNPs.
Conclusion: This study illustrated that polymorphic sites in the *CYP19A1* gene provided potential markers for selection of buffalos with better estrus behavior.

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1. Introduction

Buffalos are mainly considered as shy breeders; apart from being shy breeders, they exhibit some major reproductive constraints that result in less exploitation of their reproductive efficiency. Among the major reproductive constrains in buffalos, the expression of silent heat is the major cause of less exploitation of the reproductive efficiency of buffalos.

Silent heat is a condition in which the buffalos do not exhibit the behavioral symptoms of estrus (heat), although the physiological symptoms of estrus are present [1,2,3]. As a result of silent heat, animals become repeat breeders and fail to maintain the estrus regularity and cyclicity [4,5,6].

By studying the expressions of different genes involved in the expression of estrus and their underlying mechanisms, we can have an insight into why silent heat is most commonly observed in buffalos as compared to cows. This would help us in exploiting the reproductive efficiency of buffalos [7,8,9]. To understand the underlying mechanisms and the genes involved in controlling the estrus behavior in buffalos, the current study serves to identify novel polymorphic sites in the *CYP19A1* gene in river buffalos, which illustrated a significant association toward silent heat [10,11].

The *CYP19A1* gene in bovine species is located on chromosome 10 and was mapped to band q2.6 (Fig. 1). The gene has 10 exons, and the gene size ranges from 56 to 120 kb in different species [6,10]. It codes for the aromatase protein, which is 503 amino acids (AAs) in length. The molecular weight is 5,996,238.60 Da (GenBank Accession No. AC_000167.1). *CYP19A1* has been a very crucial responder in many reproductive pathways [5,12,13,14,15]. In this study, the *CYP19A1* gene

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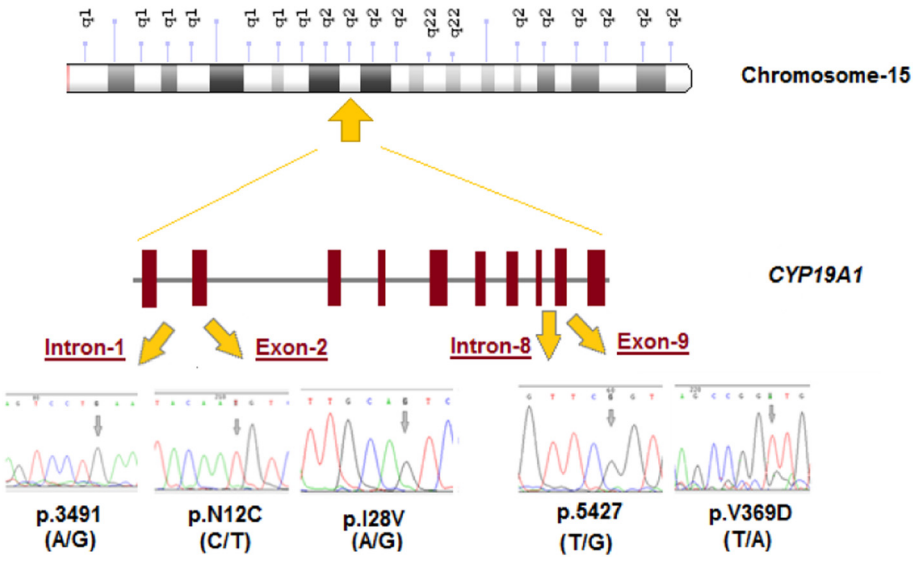

Fig. 1. Structural configuration of the *CYP19A1* gene and the identified polymorphic sites potentially associated with silent heat in Nili-Ravi buffalo.

was characterized by polymerase chain reaction (PCR) amplification and sequencing to identify genomic variants that are associated with silent estrus behavior in bovines.

2. Material and methods

The present study was conducted to identify the single nucleotide polymorphisms (SNPs) in the coding regions of the *CYP19A1* gene that affects the estrus behavior in Nili-Ravi buffalo, resulting in the poor expression of estrus behavior that ultimately leads to the silent heat or estrus condition in buffalos. The research was performed at Molecular and Genomics Laboratory, Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences (UVAS), Lahore.

2.1. Animal selection criteria

A total of 97 genetically related animals that belong to different families, showing typical phenotypic features for a given breed, were selected from several respective breeding tracts with fertility records up to 3 productive cycles from UVAS Pattoki Campus, Research Farm B and Buffalo Research Institute (BRI) Pattoki.

2.2. Sampling strategy

Animals were categorized into two groups on the basis of estrus efficiency. Estrus efficiency was determined by calculating the heat score for each individual female animal by the method reported by Roelofs et al. [15]. In this system, behavioral symptoms (urination, mucus discharge, sniffing, mounting, standing heat, etc.) of estrus after 2 to 3 intervals were recorded, and an individual score was assigned to each sign. Every time when a symptom was observed, a score was assigned to the symptom and was recorded; if the total sum of the score was equal to 50 during two consecutive observations, then the animal was considered to be showing good expression of heat signs, and if the total sum did not exceed 50 then the animal was considered to be showing low signs of heat [15]. In this context, a blood sampling was created on the basis of the most observable heat signs of estrus to obtain the required information, and animals were grouped accordingly.

Buffalos were categorized into two groups, one that showed good signs of heat and the other that showed poor signs of heat, and blood sampling was carried out in both the groups. While selecting the animals for good heat signs, season is a very important parameter. The buffalo expresses good signs of heat during the autumn season. Blood sampling for the current research was carried out during the winter season, i.e., during the months of November and December when there was no seasonal stress on animals and estrus manifestation was at its peak.

2.3. Blood sampling

Blood sampling was conducted after permission from UVAS Ethical Committee (DR/1091). Blood samples (10 ml) from each animal were collected aseptically from the jugular vein into a 50-ml Falcon tube containing 200 μl of anticoagulant, i.e., EDTA (Ethylenediaminetetraacetic acid). After the collection of blood samples, they were placed on ice and were transferred to the Molecular Biology and Genomics Laboratory, IBBT, UVAS Lahore, and were stored at −20°C before DNA extraction.

2.4. DNA extraction and quantification

DNA was extracted from the blood samples using the inorganic method of DNA extraction [12,16]. DNA quantification was done by using NanoDrop and 0.8% agarose gel electrophoresis.

Table 1
Primer sets used to amplify the partial exonic (Exons 2, 3, and 9) region of the *CYP19A1* gene.

Exons	Primer names	Sequences
Exon 2	Forward	GGGCTTGCTTGTTTTGACTC
	Reverse	CTGGTATTGAGGATGTGTCC
Exon 3	Forward	CCCAGCTACTTTCTGGGAAT
	Reverse	CTCAGGTCTCAAGCAAACC
Exon 9	Forward	TCTACGGAACAAGCACAGGA
	Reverse	GGCACGCTCAGTTTTAAGGA

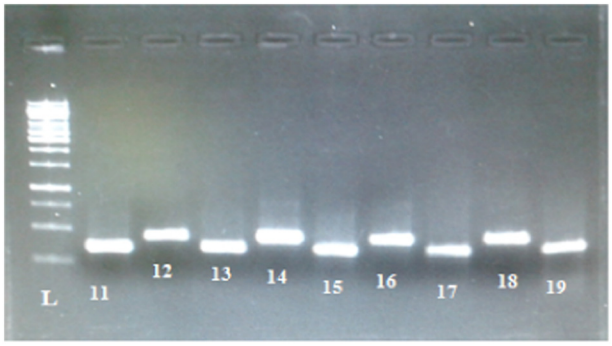

Fig. 2. PCR amplification of exons 2 and 9 in the *CYP19A1* gene.

Table 3
Chi^2 testing for Hardy Weinberg equilibrium and allelic frequency.

SNPs	Allelic frequency		$\chi 2$ ($P < 0.05$)
	A	B	
p.3491	0.1364	0.8636	0.395553
p.3550	0.3030	0.6970	0.010733*
p.N12C	0.3030	0.6970	0.530856
p.I28V	0.3333	0.6667	0.725640
p.3752	0.3939	0.6061	0.003487*
p.5415	0.2369	0.7631	0.046341*
p.5427	0.1276	0.8724	0.259965
p.5588	0.3333	0.6667	0.012673*
p.5627	0.3030	0.6970	0.398210
p.E362D	0.3933	0.6067	0.046077*
p.V369D	0.1099	0.8901	0.567830

* Non-significant.

2.5. Polymerase chain reaction

PCR was performed using the primers designed from the coding regions of the *CYP19A1* gene, i.e., exons 2, 3, and 9 (Table 1). For optimization of the primers, the Touchdown PCR protocol was followed with an annealing temperature range of 63–52°C. The reaction mixture in which the primers showed the best results was selected for the subsequent PCR. A reaction mixture of 25 μl (2.0 μl [50 ng/μl]; 10X PCR buffer 2.0 μl; Primers: forward and reverse 1.0 μl each [10 pMol]; dNTPs 2.0 μl [25 mM/μl]; $MgCl_2$ 2.0 μl [2 mM/μl]; Taq polymerase 0.15 μl [5 U/μl]; and water 14.85 μl) was used. PCR involves the following steps: 95°C × 4 min, 94°C × 30 s, 52–63°C × 45 s, 72°C × 30 s, and 72°C × 10 min. A total of 30 cycles were carried out. The PCR product containing DNA was separated through gel electrophoresis using 0.2% gel (Fig. 2).

2.6. Gel elusion and sequencing

After the amplification of the desired portion of DNA, PCR products were eluted by using FavorPrep GEL/PCR Purification Kit and loaded onto 1.2% gel to observe its quality. The PCR amplicons obtained after elusion were subjected to sequencing based on the Sanger's Chain Termination method.

2.7. Statistical and bioinformatics analyses

The sequences obtained after sequencing were analyzed using the CHROMAS software. Sequences of both groups were aligned using ClustalW software. From these aligned sequences, 11 SNPs were detected. Population genetic analysis was performed by using POPGene.32 software. The chi^2 test was performed and Hardy–Weinberg Equilibrium (HWE) was determined to find the level of significance in each loci. Association analysis was performed for those loci which were obeying HWE. The three-dimensional protein structure was also determined for CYP19A1 by using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2).

3. Results

3.1. Single nucleotide polymorphisms

In our study, a total of 11 SNPs were found in the sequence of the *CYP19A1* gene. Among these 11 SNPs, 7 were in the coding region, and 4 were in the noncoding region (Table 2). p.3491 and p.3550 were identified in intron 1; p.N12C, p.I28V, and p.3752 were identified in exon 2; p.5415 and p.5427 were found in intron 8; p.5588, p.5627, p.E362D, and p.V369D were found in exon 9 of the gene. There were four AA substitutions observed in exons 2 and 9. Of these four, only p.V369D was found to change the effect of AA substitution from non-polar to polar.

3.2. Single marker analysis

Single marker analysis provided allelic frequency and the chi^2 value of each polymorphic locus (Table 3). The *CYP19A1* gene was found homozygous for most of the polymorphic sites. Very less heterozygosity was observed throughout the sequence of the gene. Five of eleven loci were found to be significantly deviated from the HWE. The remaining loci were found to be obeying HWE with a Chi^2 value >0.05. Further, these polymorphisms were analyzed for their association by using the one-way analysis of variance (ANOVA; Mean ± SE) (Table 4). Five SNPs (p.3491, p.N12C, p.I28V, p.5427, and p.V369D) were found to

Table 2
Identified novel polymorphic sites in the *CYP19A1* gene.

SNPs	Location	Variation		Transition/transversion	Amino acid substitution	Effect
		Wild	Mutant			
p.3491	Intron	A	G	Transition	–	–
p.3550		A	C	Transversion	–	–
p.N12C	Exon 2	C	T	Transition	Asparagine>Cysteine	Slightly Polar
p.I28V		A	G	Transition	Isoleucine>Valine	Non-Polar
p.3752		C	T	Transition	Alanine	–
p.5415	Intron	A	G	Transition	–	–
p.5427		T	G	Transversion	–	–
p.5588	Exon 9	C	G	Transition	Proline	–
p.5627		T	C	Transition	Aspartate	–
p.E362D		G	T	Transversion	Glutamic acid>Aspartate	Polar
p.V369D		T	A	Transversion	Valine>Aspartate	Non-Polar > Polar

Table 4
Association analysis of polymorphic sites with silent estrus behavior.

SNPs	Association analysis (n = 97)			Probability ($P < 0.05$)
	AA	AB	BB	
p.3491	n = 35 53.95 ± 0.5233	n = 06 42.9 ± 0.1307	n = 56 17.475 ± 0.2529	<0.0001
p.3550	n = 94 45.83 ± 0.7667	n = 03 25.6 ± 0.4743	–	1.00000
p.N12C	n = 25 54.87 ± 0.67	n = 12 25 ± 0.9103	n = 60 17.6 ± 0.1472	<0.0001
p.I28V	n = 35 55.5 ± 0.0913	n = 09 46.9 ± 0.5102	n = 53 17.625 ± 0.1548	<0.0001
p. 3752	n = 22 60.55 ± 0.4555	–	n = 75 16.12 ± 0.1315	1.00000
p.5415	n = 35 54.37 ± 0.1109	–	n = 62 16.5 ± 0.2309	1.00000
p.5427	n = 15 54.375 ± 0.1109	n = 10 34.46 ± 0.8253	n = 72 14.5 ± 0.2309	<0.0001
p.5588	n = 22 54.37 ± 0.1109	–	n = 75 14.5 ± 0.2309	1.00000
p.5627	n = 36 54.52 ± 0.1685	–	n = 61 14.26 ± 0.1764	1.00000
p.E362D	n = 42 53.72 ± 0.4652	–	n = 55 18.93 ± 0.1764	1.00000
p.V369D	n = 21 53.72 ± 0.4652	n = 15 31.43 ± 0.2925	n = 61 18.95 ± 0.1642	<0.0001

have a strong association with poor heat score ranging from 14.5 to 18.95 (Fig. 1).

3.3. Three-dimensional protein structure

A three-dimensional protein structure was constructed to locate the exonic polymorphisms. The CYP19A1 protein carries various functionally significant regions. The SNP p.V369D was found in the steroid-binding domain, inclining the protein activity toward the more polar side (Fig. 3). The SNP p.I28V was located as a part of the transmembrane helix of the protein in the mitochondrial membrane (Fig. 4). These mutations describe the variation in protein binding and activity, showing that the aromatase sequence has been altered because of these mutations, thereby causing silent estrus behavior.

4. Discussions

The SNPs identified in intron 1, exon 2, intron 8, and exon 9 were analyzed, and five of them were found to have a strong association with the estrus score of each buffalo. The *CYP19A1* gene has previously been studied for its role in endocrine regulation of reproductive hormones and identification of different polymorphic sites [16,17,18]. It clearly depicts the polymorphic nature of the gene, especially its tendency toward estrus cyclicity. Four SNPs in the *CYP19A1* gene in the Murrah buffalo have been reported by Suneel et al. [19]. SNPs found in the current research are not previously reported in any study so these are novel for Nili-Ravi buffalo. The SNPs reported by Suneel et al. [19] were in the intron 3, which was a transition, and intron 7, which was a transversion, and they were not in the splice junctions. Similarly, the SNPs identified in the current research in the intronic region did not have any effect on the splice junctions, binding sites,

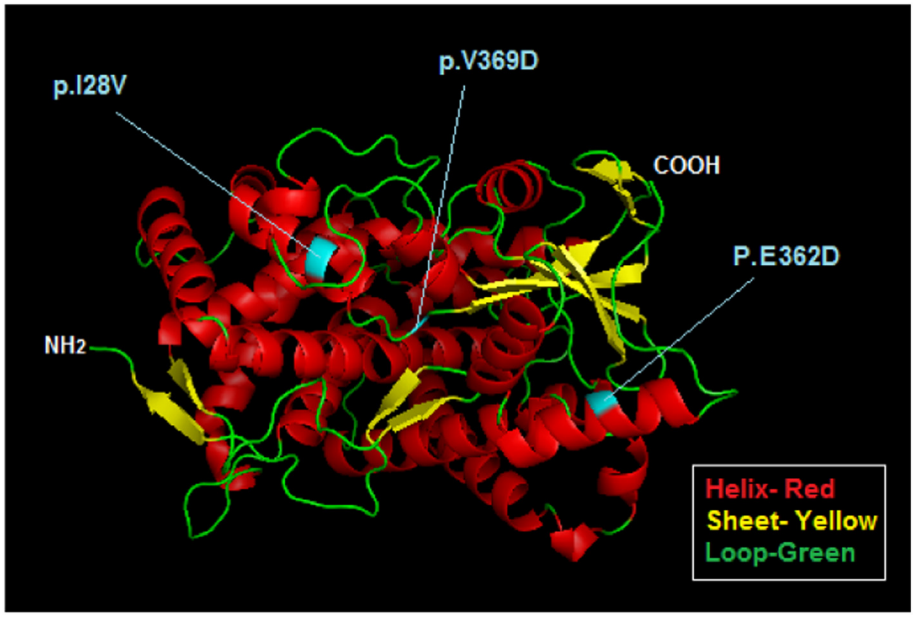

Fig. 3. Three-dimensional predicted protein model of CYP19A1 by homology modeling.

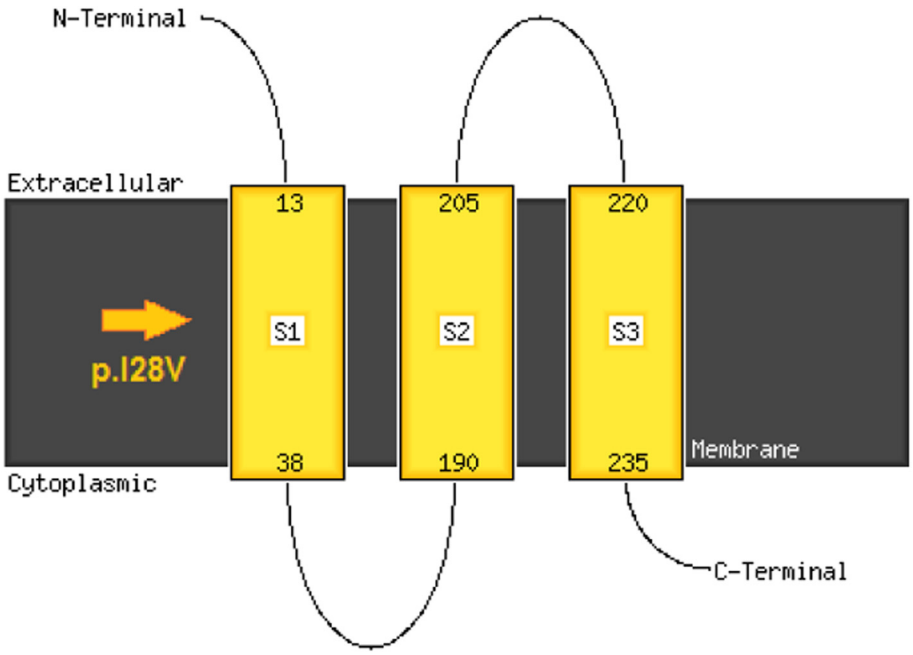

Fig. 4. Transmembrane helix of the CYP19A1 protein illustrated location of p.I28V.

and transcription machinery. The SNPs reported in the noncoding regions, i.e., the intronic regions, can encode for microRNA responsible for RNA-mediated gene silencing through interference techniques. SNP in the exon 2 region of the *CYP19A1* gene, which was a transversion, was reported by Suneel et al. [19] as compared to the SNP identified in the exon 2 region of the *CYP19A1* gene in the current research. SNPs in the *CYP19A1* gene have also been reported by Cochran et al. [1] for controlling the silent estrus behavior in Holstein cows. SNPs in the promoter 1.2 of the bovine *CYP19A1* gene have been reported by Vanselow et al. [18] by selecting six different animal breeds, i.e., Belgian Blue-White, German Angus, Galloway, Uckermark, Red and White Cattle, and German Holstein. The SNPs identified by Vanselow et al. [18] were both transitions. SNPs in the *CYP19A1* gene have also been reported by Cho et al. [6], and these authors demonstrated that these mutations cause an increase or decrease in the aromatase activity, thereby altering the levels of the circulating estrogen. Two novel SNPs in the promoter 2 and intron 9 regions of the *CYP19A1* gene in five sheep breeds, i.e., British Milk Sheep, Carranzana, Latxa Black Face, Latxa White Face, and Merino, have been reported by Zsolnai et al. [20]. The highly polymorphic nature of the gene proposes it as a pretentious candidate gene for identification of selection signature that can be useful in future breeding programs for superior animals with better reproductive efficiency. In the past, large improvement has been made in dairy animals, especially buffalo and cow, to increase their milk production, which results in affecting their fertility rate, and there is also a negative association between milk trait and fertility in buffalo and cow. As a result, the reproductive capabilities remain unexploited also because of the improvement in buffalo for milk traits and because of some major reproductive constraints such as the expression of silent estrus. Keeping in view this constraint, the current research was based on the candidate gene analysis that has a profound effect on the silent estrus behavior in Nili-Ravi buffalo.

5. Conclusions

It can be concluded from the above discussion that the SNPs identified in the current research can be used as genetic markers for the silent estrus, a major reproductive constraint in the Nili-Ravi buffalo. These markers can be used in genetic evaluation or for the selection of breeds.

Competing interests

There is no conflict of interest for this manuscript.

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