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Identification of *Pit1* Gene Variants in Sahiwal Cattle of Pakistan

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Authors' Contribution
AN conceived and designed the study. AN,TH did sampling and genome extraction. SM, MJ,SM amplified the markers. MEB, MJ, WS, RZI analyzed the data. AN wrote the article.

Key words
Bovine Pit1 Gene, Variants, Heterozygosity, Cattle, Pakistan.

ABSTRACT

Pit-Oct-Unc Domain, Class 1, Transcription Factor 1 (Pit1) is a member of the tissue-specific POU homeobox transcription factor family that is found in all mammals. Pit1 encodes a pituitary-specific transcription factor, which is involved in pituitary development and regulating the hormone expression in animals. In current study, *Pit1* gene was screened for polymorphic sites in Sahiwal cattle. Samples from Sahiwal cattle breeds were sequenced using six set of primers. A total 15 polymorphisms; 12 in intronic and 3 in exonic region, were identified. The sequences of the amplified *Pit1* gene fragments were aligned with the help of BLAST for SNPs identification. This is a first report toward genetic screening of this gene at molecular level in Sahiwal cattle of Pakistan. No work has been reported on this gene in Sahiwal cattle. In this study, a new set of single nucleotide polymorphisms (SNPs) were reported. Heterozygosity, allelic and genotypic frequencies of identified variants were also determined. Chi^2 test was performed to evaluate the Hardy-Weinberg Equilibrium of each polymorphic site. Two loci were found to be in HWE. These identified alleles will be useful for animal selection at molecular level *i.e.* an ideal tool for marker-assisted selection of animals for future breeding programs.

INTRODUCTION

Pit-Oct-Unc Domain, Class 1, Transcription Factor 1 (Pit1), also known as POU1F1, is involved in a variety of signaling pathways that are important for many developmental and physiological processes including pituitary gland development, mammary gland development and growth, milk protein expression and milk production and secretion. It is considered to be a candidate gene for the regulation of growth and development in cattle and other mammals (Zhang *et al*., 2009; Majeed *et al*., 2013). It is crucial for the expression of GH, Prolactin, and TSH β-subunit in mammals. Moreover, binding of growth hormone and prolactin to their receptors on the cell membrane triggers a cascade of signaling events including the JAK/STAT pathway, which has been shown to be required for adult mammary gland development and lactogenesis (Mullis, 2007; Huang *et al*., 2008; Herman *et al*., 2012).

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The gene is located on the chromosome 1 of *Bos taurus* (cattle), and spanning 15952 base pairs comprising 6 exons and 5 introns. A 33 kDa Pit1 transcription factor protein has two important regions for the transcriptional regulation of target promoters (Bastos *et al*., 2006). The changes in nucleotide sequence in the gene may lead to altered function of the product. Previously, no study was conducted to identify the SNPs (Single Nucleotide Polymorphisms) in Pakistani cattle breeds. So, the objective was to identify the SNPs and to determine the allelic and genotypic frequency of this gene in Pakistani Sahiwal cattle using DNA sequencing approaches, which may have a significant effect as genetic markers for milk production performance traits.

MATERIALS AND METHODS

The present study was conducted to identify genetic variants and to determine its heterozygosity, allelic and genotypic frequency in Sahiwal dairy cattle breed of Pakistan.

Animal blood samples and DNA extraction

Fifty true representative unrelated cows from Sahiwal

cattle breed was selected from various Government Dairy and Livestock Farms. Blood samples were collected from the Jugular vein of each animal into tubes containing 200 µL Ethylene Diamine Tetraacetic Acid (0.5 M EDTA).

PCR amplification and DNA sequencing

Genomic DNA was extracted from the blood samples according to Phenol-Chloroform DNA extraction method (Sambrook and Russel, 2001). For the quantification of DNA, 0.8 % agarose gel was used and visualized under UV light.

Primers were designed from NCBI Gene Bank Accession No. NC_007299.3, *Bos taurus* chromosome 1, reference assembly (based on Btau_4.0), whole genome shotgun sequence (Elsik *et al*., 2009), using online software Primer3 (http://frodo.wi.mit.edu/). Primer specificity was also checked by using UCSC genome browser (http://genome.ucsc.edu) in-silico PCR (http://genome.uscs.edu/cgi-bin/hgPer).

DNA samples were amplified following optimization of primers. The PCR reaction mixture in a volume of 25µL using 50ng/µL genomic DNA, 10x PCR buffer, 2.5 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 U *Taq* polymerase, and 0.5 µL of each primer. Touchdown PCR profile had an initial denaturation at 95°C for 4 min, 35 cycles of 94°C (30s), 64-54°C (45s), 72°C (45s) and final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis using aliquot (2 µL) of each amplified product in 1.2% agarose gel stained with ethidium bromide for 30 min and visualized under UV light using digital camera apparatus (BioRad). PCR products were purified using Favor Prep™ Gel/PCR purification kit to recover/concentrate DNA Fragments from agarose gel (http://www.favorgen.com/products_for_res_nae_FAGCK.htm). POU1F1 gene PCR amplified products were precipitated and sequenced on ABI 3100 DNA sequencer.

Bioinformatics analysis

Multiple amplifications from the original samples were done. All the sequences were aligned with the help of NCBI online software blast-2-sequence (http/www.ncbi.nlm.nih.com). SNPs were identified from the aligned sequences. BioEdit translate tool was used to analyze SNPs to look for changes in codons and hence amino acids and protein sequence. All polymorphic positions were analyzed to determine allelic and genotypic frequencies using online software SNPator (http://www.snpator.org).

RESULTS AND DISCUSSION

Sequence analysis revealed fifteen polymorphic sites in amplified exonic and associated intronic region of *Pit1* gene of Pakistani Sahiwal cattle breed. Out of these fifteen SNPs, three were identified in exonic and twelve in intronic region (Table I).

Data of allele distribution, allele and genotype frequencies clearly indicating that dimorphic alleles obeyed assumption of the Hardy-Weinberg equilibrium demonstrating that alleles are randomly distributed throughout the population, no migration has occurred, no

Table I.- Allelic distribution of identified variants.

Sr. No.	Chromosomal position	Nucleotide change	Template	H. observed	H. expected	P. value
1	35752429	G→A	Intronic	0.0645	0.4870	0.0013**
2	35752472	G→A	Intronic	0.0968	0.4979	0.0011**
3	35752554	G→A	Intronic	0.0323	0.4979	0.0010**
4	35752568	G→A	Intronic	0.0645	0.4953	0.0015**
5	35752576	G→A	Intronic	0.0645	0.4745	0.0014**
6	35752636	G→A	Exonic	0.0968	0.4813	0.0012**
7	35752638	G→A	Exonic	0.1290	0.2248	0.2016
8	35752650	G→C	Intronic	0.0968	0.5000	0.0014**
9	35752653	A→C	Intronic	0.0323	0.4813	0.0011**
10	35752658	G→C	Intronic	0.0645	0.4870	0.0017**
11	35752665	T→G	Intronic	0.0968	0.4480	0.0001**
12	35752813	C→A	Intronic	0.0645	0.4953	0.0019**
13	35753683	T→A	Intronic	0.0323	0.4979	0.0000**
14	35756143	C→A	Exonic	0.1613	0.2919	0.1097
15	35756263	G→A	Intronic	0.0645	0.4579	0.0021**

* P-value <0.05; ** P-value <0.01.

bottlenecks happened and population remained large in numbers. While probability (P-value) of Chi-square test at all other positions is less than 0.05 (Tables I, II).

Table II.- Allelic and genotypic frequencies of identified variants.

Sr. No.	Chromosomal position	Allele frequency		Genotype frequency		
1	35752429	A	G	AA	AG	GG
		0.5806	0.4194	0.5484	0.0645	0.3871
2	35752472	A	G	AA	AG	GG
		0.5323	0.4677	0.4839	0.0968	0.4194
3	35752554	A	G	AA	AG	GG
		0.5323	0.4677	0.5161	0.0323	0.4516
4	35752568	A	G	AA	AG	GG
		0.5484	0.4516	0.5161	0.0645	0.4194
5	35752576	A	G	AA	AG	GG
		0.6129	0.3871	0.5806	0.0645	0.3548
6	35752636	A	G	AA	AG	GG
		0.5968	0.4032	0.5484	0.0968	0.3548
7	35752638	A	G	AA	AG	GG
		0.8710	0.1290	0.8065	0.1290	0.0645
8	35752650	C	G	CC	CG	GG
		0.5000	0.5000	0.4516	0.0968	0.4516
9	35752653	A	C	AA	AC	CC
		0.4032	0.5968	0.3871	0.0323	0.5806
10	35752658	C	G	CC	CG	GG
		0.5806	0.4194	0.5484	0.0645	0.3871
11	35752665	G	T	GG	GT	TT
		0.6613	0.3387	0.6129	0.0968	0.2903
12	35752813	A	C	AA	AC	CC
		0.4516	0.5484	0.4194	0.0645	0.5161
13	35753683	A	T	AA	AT	TT
		0.4677	0.5323	0.4516	0.0323	0.5161
14	35756143	A	C	AA	AC	CC
		0.8226	0.1774	0.7419	0.1613	0.0968
15	35756263	A	G	AA	AG	GG
		0.3548	0.6452	0.3226	0.0645	0.6129

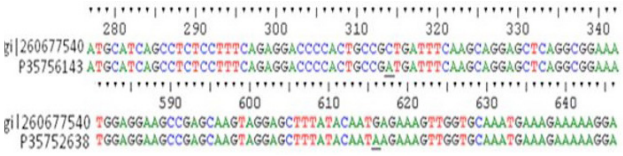

Fig. 1. Amino acid codon changes of subject *vs.* reference protein.

Those SNPs were further analyzed for their codon change and translated to their corresponding amino acids. Nucleotide change at P35752638 is from G→A. This nucleotide changes the amino acid codon from GAG→AAG (Fig. 1) indicating an amino acid substitution from glutamic acid (E) to lysine (K) (Fig. 2). As glutamic acid is an amino acid of acidic nature whereas lysine is basic. The opposite nature of both amino acids is a clear indication that the nature of final protein product of the gene was changed. At P35756143 the C→A change of nucleotide, changes the codon from GCT→GAT, causing an amino acid substitution from alanine (A) to aspartic acid (D). Alanine is a non-polar amino acid while aspartic acid is acidic polar. Being opposite in nature, the final protein product of the gene should be change in nature (Table III). Both polymorphic sites are very important because the nature of substituted amino acids changed due to the substituted nucleotide at these sites. A vertical bar chart showing percentage composition and nature of reference and subject protein are shown in Figure 3. These SNPs may serve as a powerful genetic resource for the development of DNA markers for association studies. The comparison of the amino acid composition of reference protein and the protein having these amino acid changes shows that the total length of the protein remains the same *i.e.* 291 amino acids. But the molecular weight changed from 33036.97 Dalton (reference protein) to 33080.04 Dalton (Changed protein). This also indicates the change in nature of the final protein product of the gene.

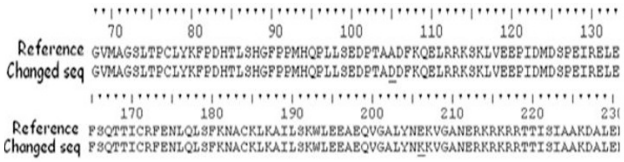

Fig. 2. Glutamic acid (E), lysine (K), alanine (A) and aspartic acid (D) substitution of subject *vs.* reference protein.

Table III.- Synonymous and non-synonymous changes in exonic region

Chromosomal position	Codon change	Amino acid change	Change type
35752636	GAG → GAA	Glutamic acid	Synonymous
35752638	GAG → AAG	Glutamic acid → Lysine	Non-synonymous
35756143	GCT → GAT	Alanine → Aspartic acid	Non-synonymous

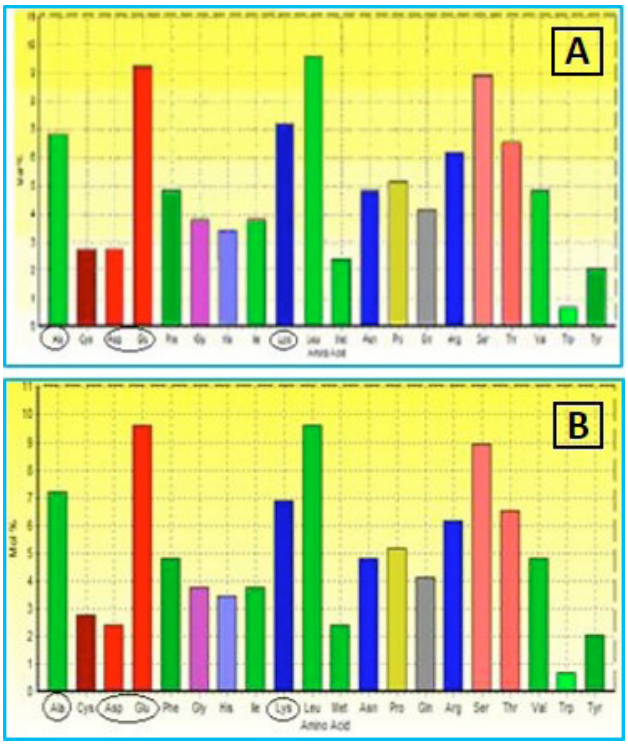

Fig. 3. Bar chart of amino acid composition of subject (changed) protein (A) and reference protein (B).

Thus this bovine *POU1F1* (*Pit1*) gene was considered as one of the important candidate gene to describe the genetic variability and marker assisted selection programs in various breeds. Fontanesi et al. (2015) genotyped the g.35009083 G>A polymorphism in *POU1F1* gene and analyzed its association with milk production traits in Reggiana breed. A point mutation (A→G) in exon-6 was found to have affected on HinfI restriction site (Woollard et al., 1994). The association test with milk yield and conformation traits was performed on Italian Holstein-Frisians bulls (Renaville et al., 1997), Piemontese cattle (Di-Stasio et al., 2001), Nanyang cattle (Xue et al., 2006).

The 545 G→A substitution was identified in exon 2 while 577 C→A; 606 T →C; 647 A →G substitutions in exon 3 were reported in two North American Holstein cattle resource populations. Among these mutations, only one SNP was a missense mutation *i.e.* 577 C →A, induced the proline to histidine substitute into final protein product. Herman et al. (2012) analyzed the significant association of POU1F1 variants with milk yield and productive life. Genotype analysis and allele frequency has also been carried out several times and its association with milk production in different cattle breeds were also reported (Zakizadeh et al., 2007; Han et al., 2010). This gene was also characterized in local Azakheli buffalo population (Nadeem et al., 2013). It was not considered a candidate gene with production traits only, Wang et al. (2015) investigated the effect of the *Pit1* gene on meat quality traits in the Hyla, Champagne, and Tianfu Black rabbit breeds and reported that POU1F1 SNP (at 536 bp in intron 5) may be of potential use in marker assisted selection (MAS) for meat quality traits in rabbits.

CONCLUSION

To conclude, the present study was an example of candidate gene approach to find novel variations at DNA level. All identified alleles will be useful for animal selection *i.e.* an ideal tool for marker-assisted selection of animals for breeding. Because of the lack of functional data and small population size used, the conclusion may be helpful for further studies on the effect of this gene with different productive and reproductive traits in cattle breed populations.

Statement of conflict of interest

Authors have declared no conflict of interest.

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