Journal of Animal Research: v.7 n.6, p. 993-1000. December 2017

DOI: 10.5958/2277-940X.2017.00150.4

Allele Fixation in Prolactin and Pit-1 Genes Associated with Highest Fat Content than Milk Yield of Murrah Buffalo Population

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Received: 07 Sept., 2017 **Revised:** 12 Dec., 2017 **Accepted:** 14 Dec., 2017

ABSTRACT

Present study was focused on the genetic variability in Prolactin and Pituitary specific transcription factor-1 genes using Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) and nucleotide sequencing techniques. DNA was isolated from 100 Murrah buffaloes maintained at directorate of livestock farms, GADVASU Ludhiana. A 294 bp fragment of PRL and 451 bp fragment of Pit-1 gene was amplified and digested using *Rsa*I and *Hinf*I restriction enzymes. The genotypes were then sequenced using sanger di-deoxy chain termination method and were compared with related species. Both *Rsa*I and *Hinf*I locus showed only one genotype. The amplicons of both genes exhibited absence of polymorphism with respect to restriction enzymes used and accordingly, the allelic frequency was found to be unity. The monomorphic pattern of the amplicon of these genes with respect to different enzymes indicated the concernedness of these genes. Percent diversity and divergence matrix analysis revealed that both genes are in close cluster with swamp buffalo and *bos species* as compared to other species included in the sequence comparison. The result suggested that the allele has been fixed in the herd and need to inclusion of the Murrah buffalo from other population in the herd to introduce desired genetic variability to enhance the milk production. Sequence comparison of PRL and Pit-1 genes of Murrah buffaloes with other species is little divergent.

Keywords: PRL, Pit-1, Murrah Buffalo, PCR RFLP, sequencing

The buffalo is considered as an important milch animal in India as more than 60 per cent of the total milk produced is buffalo milk. The average milk productivity of buffaloes in India is much higher (4.30 Kg/day/animal) than indigenous cattle (1.97 kg/day/animal) which revealed the importance of buffalo as compared to indigenous / non-descript cattle. The contribution of buffaloes to the total milk production of India (110 million tonnes) is around 56%. Chromosomes contain three genes PRL, Pit-1 and GH in anterior pituitary cell. Bovine prolactin (PRL) gene is localised in chromosome 23 and consists of five exons separated by interval introns. Boleckova *et al*. (2012) reported the polymorphic G allele of bPRL which was positively associated with milk yield ($P < 0.05$) and also positively influenced protein ($P < 0.05$) and fat yields ($P < 0.01$). Dong *et al.* (2013) reported that Chinese Holstein cows with AA at locus 7545 had a higher milk yield at 305 days (8457 ± 938 kg) than cows with GA (7537 ± 1278 kg; $P < 0.01$) or GG (7757 ± 1174 kg; $P < 0.05$). Alipanah *et al.* (2007) were also of the opinion that AA genotype had a positive effect on milk yield in Russian Red Pied cattle. Digestion of prolactin gene with *Rsa*I restriction enzyme revealed two alleles A and B and BB, AB and AA genotype had higher lactation length, higher lactation yield and shorter service period, respectively (Mahajan *et al*., 2012). Dybus (2002) showed that cows with the AA genotypes of the PRL gene had higher milk protein content than AB individuals.

Pit-1 polymorphism was found to be associated with milk yield and conformation traits in cattle (Renaville *et al.,* 1997). Doosti *et al*. (2011) reported that AA genotypes of

the Pit-1/*Hinf*I genotype could be useful in fertility and create the next generation for increase in milk production and growth of Holstein cattle. Cosier *et al.* (2012) reported polymorphism in relation to important milk yield, fat and protein percent and confirmed this locus as the candidate genes that may produce differences in milk characteristics and can be used in marker assisted selection in Romanian Simmental cattle. Zakizadeh *et al.* (2007) found the transition from A to G in nucleotide 1256 responsible for *Hinf*I allele. Few reports are available for polymorphism in Pit-1 gene for buffaloes (Verma, 2013) and no reports are available on the association of Pit-1 with economic traits in Murrah buffaloes till date. Therefore, present study was conducted to access the genetic variability in PRL and Pit-1 gene of Murrah buffaloes so that, promising DNA markers could be developed to improve milk production and milk constituents traits in Murrah buffaloes.

MATERIALS AND METHODS

Prior permission to conduct the experiment was taken from Institutional Animal Ethical Committee (IAEC, Registration no. 497/GO/Re/SL/01/CPCSEA), Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

Blood Collection and DNA extraction

Blood samples were collected in 0.5% EDTA from 100 Murrah buffaloes and DNA was extracted according to phenol-chloroform extraction method (Sambrook and Russell, 2001) with minor modifications. Quality and quantity of DNA was determined by spectrophotometer at optical density of 260 and 280 nm.

Amplification of gene

Exon 3 of PRL gene and exon 6 of Pit-1 gene were amplified using primers mentioned in Table 1.

Optimization of PCR reaction mixture

The reaction mixture was optimized using variable concentrations of different components for amplification of specific regions of PRL and Pit-1 genes in a final volume of 25 µl. The optimized PCR reaction mix that was finally used for amplification is as follows (Table 2).

Table 2: The optimized PCR reaction mixture

Sl. No.	Reaction components	Volume (µl)	Final Con.
1	Autoclaved distilled water	10.5	—
2	10 X PCR buffer	2.5	1.5 mM
3	dNTP mix	2.0	0.2 mM
4	$MgCl_2$	1.5	1.5 mM
5	Forward primer	1.0	30 pmol/µl
6	Reverse primer	1.0	30 pmol/µl
7	Taq DNA polymerase	0.5	5 U/µl
8	Genomic DNA	5	80-100 ng
9	DMSO	1	
	Total	**25 µl**	

PCR Digestion of Prolactin and Pit-1 genes

Digestion of PCR products was carried out using *Rsa*I and *Hinf*I (invitrogen) restriction enzyme for Prolactin and Pit-1 genes respectively. In brief, 5 µl autoclaved distilled water, 2 µl10 × buffer T for RE, 1 µl restriction enzyme (10U/µl), 2µl 0.1% BSA and 10 µl PCR product and incubated for 4hrs at 37°C in water bath. The digested products were run on agarose gel from 2 – 4% as expected size of fragments with suitable DNA marker.

Sequencing of PCR products

The products obtained by PCR were then cleaned to remove salts and excess primer by QIAquick Gel Extraction Kit

Table 1: Primers for amplification of target region of PRL and Pit-1 genes

Sl. No.	Name of the gene	Primer Sequence 5'_____3'	Annealing temp	Size and location	Restriction enzyme	References
1	Prolactin	CCAAATCCACTGAATTATGCTT ACAGAAATCACCTCTCTCATTCA	58°C	294 bp Exon-3	*Rsa*I	Brym *et al.,* 2005
2	Pit-1	AAACCATCATCT CCCTTCTT AATGTACAATGTGCCTTCTGAG	56°C	451 bp Exon-6	*Hinf*I	Renaville *et al.,* 1997

Table 3: PCR programme for amplification

Sl. No.	Steps		Temperatures for amplification of fragments of PRL and Pit-1 genes		
			294bp	451bp	Time
1		Initial denaturation	94 °C	94 °C	5 min
2	35 cycles	Cyclic denaturation	94 °C	94 °C	1 min
		Cyclic annealing	55°C	56°C	1 min
		Cyclic extension	72 °C	72 °C	1 min
3		Final extension	72 °C	72 °C	10 min
4		Storage	4°C	4°C	Forever

Protocol (using a micro centrifuge) purification kit. A 25µl of PCR product was mixed with equal amount of binding buffer incubated for a minute and the mixture was then transferred to QIAquick column and centrifuged at 10000 rpm for 1 min, 0.75 ml of wash buffer was then added on the QIAquick column and centrifuged again at 13000 rpm for 1 min (one more spinning of empty column may be required for removal of remaining wash buffer). The solution was finally eluted by adding 50 ul of elution buffer and collected in 2.0 ml micro-centrifuge tube. The representative products obtained were then sequenced by Sanger dideoxy chain termination method and the sequences so generated were subjected to BLAST (www.ncbi.nlm.nih.gov/BLAST) analysis to ascertain that sequences were of PRL and Pit-1genes and submitted to database. The corresponding nucleotide sequences were retrieved from NCBI database and were aligned using DNASTAR software.

RESULTS AND DISCUSSION

PCR amplification of target regions of PRL and Pit-1 genes

The regions of PRL and Pit-1 genes were amplified using different sets of primers by Polymerase Chain Reaction (PCR). The PRL and Pit-1 region comprised of exon 3 and 6 were sequenced, respectively. The sequencing results revealed that 294 bp PRL and 451 bp Pit 1 fragments consists of 179 bp long exon 3 and 208 bp long exon 6, respectively. Both were analyzed and submitted to GenBank under the accession No.KT229547 and KT160273, respectively.

PCR-RFLP of 294 bp fragment

The amplified fragment digested with *Rsa*I (GT↓AC) produced one band of 294 bp which was confirmed by the presence of cutting site at 294th position in the sequence. This type of banding pattern suggested that amplified fragment corresponding to exon 3 contained only one RE site for the restriction enzyme in all the tested animals. No polymorphism was found with respect to these restriction enzyme and the gene and genotype frequency for these fragment were calculated to be 1.00. So, the prolactin gene fragment of 294bp corresponding to exon 3 was found to be monomorphic with respect to *Rsa*I RE site (Fig. 1).

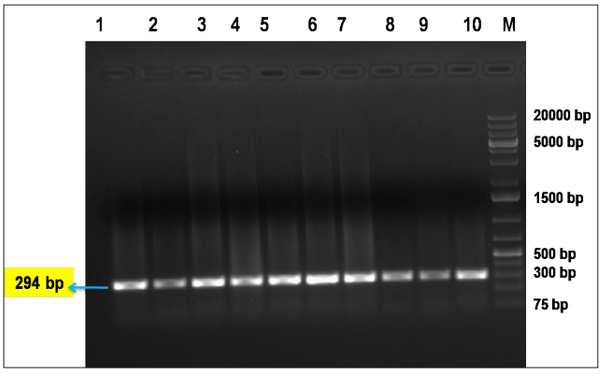

Fig. 1: PCR-RFLP electrophoresis pattern for Prolactin gene, Lane M: 100 –bp marker, Lane 1-8: undigested 294 bp fragment (Homozygous GG genotypes)

All the buffaloes investigated in the present study are genotyped as GG homozygous genotype. GG genotype is already reported to be associated with high fat % and growth and low milk production in buffaloes (Brym *et*

al., 2005; Othman *et al.,* 2011) in comparison to AA and AG genotypes in buffaloes. Exon 3 of prolactin gene in buffalo population of Khuzestan-Iran was found to be monomorphic (Tabar *et al.,* 2010). In contrast to it, AA (162bp, 132bp) and AG genotypes yielded more milk fat than BB genotype in cattle (Dybus *et al*., 2004; Khatami *et al.,* 2005) whereas the highest milk, milk fat and milk protein were obtained by cows with GG genotype (Alipanah *et al.,* 2007). Das *et al*. (2012) found that PRL gene exon 3, genotype BB was associated with highest lactation milk yield (1007.354 ± 92.328 kg) whereas heterozygotic genotype AB was associated with highest fat percentage (4.780 ± 0.126) and highest protein % in Deoni cattle.

Characterization of prolactin gene

The summary of nucleotide variation among different species with respect to exon 3 of 294 bp fragment presented in Table 4. The sequence of 294 bp fragment of PRL gene of Murrah buffaloes was compared with similar sequence of other ruminants viz. *Bos indicus*, *Bos taurus*, *Bubalis bubalis* (swamp), *Capra hircus* and *Ovis aries*. On comparison of Murrah buffalo with swamp buffalo, no nucleotide variation could be noticed in exon 3. Though with *Bos indicus*, five nucleotide variations were observed in the exon 3 at 59th (G→C), 60th (A→G), 97th (C→T), 127th (A→C) and 133rd (G→A). Three nucleotide differences have been noticed, when Murrah buffalo sequence was compared to *Bos taurus* at 97th (C→T), 127th (A→C) and 133rd (G→A). Similarly, when Murrah buffalo was compared with goat and sheep, one nucleotide variation was found in 150th (C→T) position. The estimates of genetic relatedness (similarity and divergence) for nucleotide and amino acid have been presented in Fig 2. The nucleotide sequence homology of Murrah buffalo was found to be highest (100%) with swamp buffalo, while it was lowest (62.6%) with *Ovis aries*.

Analysis of deduced amino acid revealed that exon 3 coded for 60 amino acids (Fig 3). The deduced amino acid sequence of exon 3 of PRL of Murrah buffalo was compared with similar sequence of other species viz. *Bos indicus*, *Bos taurus*, *Bubalis bubalis* (swamp) *Capra hircus* and *Ovis aries*. The absolute similarity of Murrah with swamp buffalo and *Bos taurus* was reflected in their deduced amino acid sequence. *Bos indicus* exhibited little variation in deduced amino acid sequence due to incomplete sequence with respect to Murrah buffalo. However, one difference was observed with respect to sheep and goat at 32th position (Alanine→Valine) in exon 3. The deduced amino acid sequences showed 100% homology with swamp buffalo and less similarity was noticed with sheep and goat (98.3%).

Table 4: Summary of nucleotide variations corresponding to exon 3 of PRL gene

	Exon 3 (from 56 to 235)					
Nucleotide Position #	**59**	**60**	**97**	**127**	**133**	**150**
Buffalo (Murrah)	G	A	C	A	G	C
Buffalo (Swamp)	G	A	C	A	G	C
Bos indicus	**C**	**G**	**T**	**C**	**A**	C
Bos Taurus	G	A	**T**	**C**	**A**	C
Capra hircus	G	A	C	A	G	**T**
Ovis aries	G	A	C	A	G	**T**

Nucleotide position as per Buffalo sequence i.e. Consensus sequence

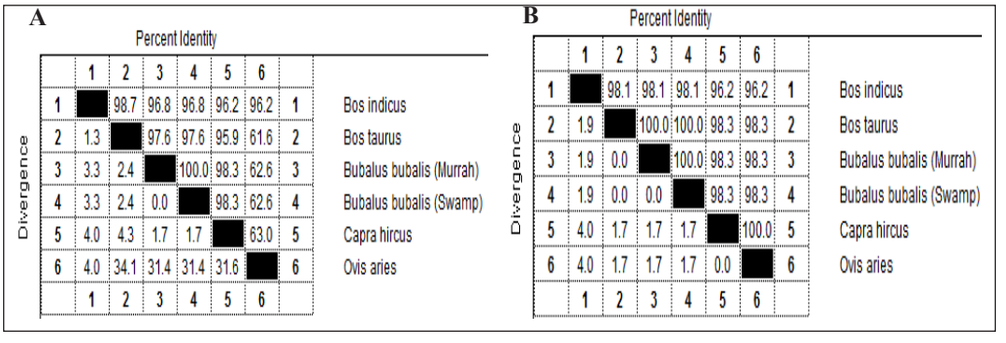

Fig. 2: Sequence homology on the basis of Nucleotide (A) and Amino Acid (B) sequence of PRL gene (294bp)

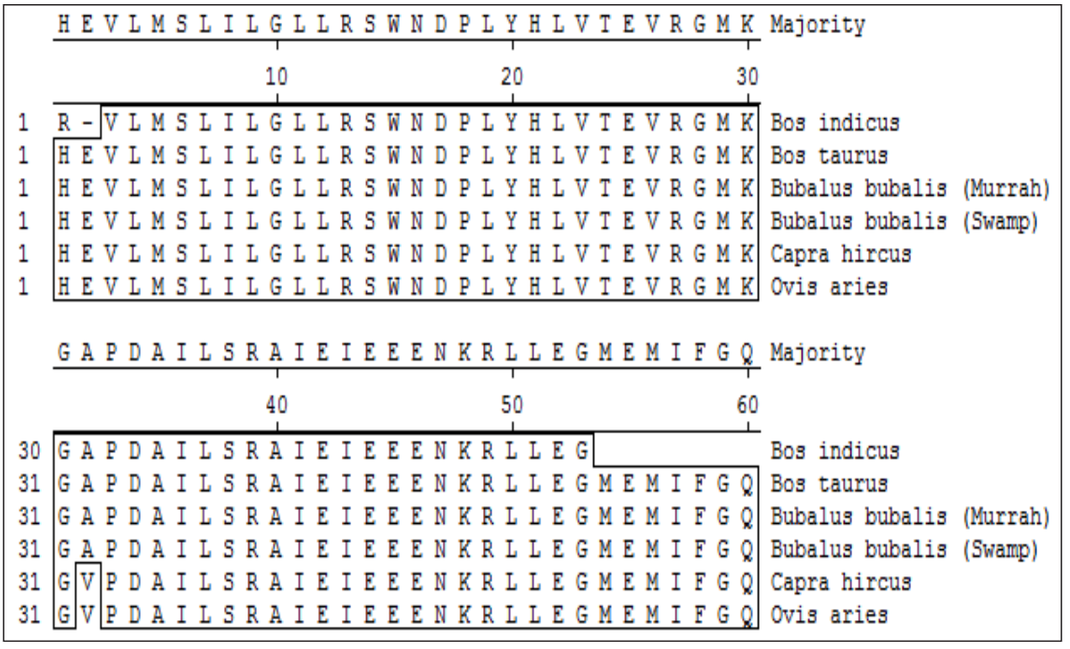

Fig. 3: Comparative analysis of amino acid sequence of PRL gene (60bp fragment)

PCR-RFLP studies on 451 bp fragment

Digestion of this amplicon with *Hinf*I (G↓ANT) produced two bands of 244 bp and 207 bp which was confirmed by the presence of restriction site in the sequence. All the bands were clearly visible in the gel. This type of banding pattern suggested that 451bp amplified fragment contained only one RE site for *Hinf*I restriction enzyme.

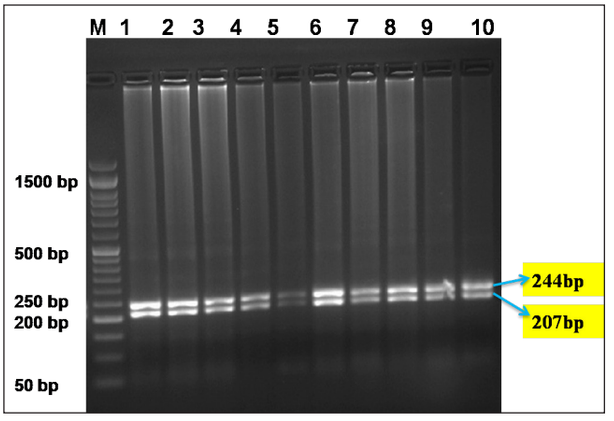

Fig. 4: The PCR-RFLP electrophoresis pattern for Pit-1 gene, Lane M: 50 –bp marker, Lane 1-10: digested 244 bp and 207bp fragment (Homozygous BB genotypes)

No polymorphism was found with respect to this restriction enzyme in the animals under study and the gene and genotype frequency for these fragments were calculated to be 1.00. There is no PCR-RFLP report for this particular fragment also, till date for Murrah buffalo. All the Murrah buffaloes in the present study are genotyped as BB homozygous genotype where the amplified fragments of all tested DNA samples were at 451-bp and digested with *Hinf*I endonuclease giving two digested fragments at 244-bp and 207-bp (Fig 4) Similar reports were given by Misrianti *et al.* (2010) in which he found no polymorphism in Pit-1/*Hinf*I locus in Indonesian buffaloes.

The overall genotype frequency for Pit-1 gene was BB (100%). Dybus, *et al.* (2004) found frequency of B allele 0.74 and 0.75 in Polish Black-and-White cattle as compared to frequency of A allele. But in contrast to it, Hori-Oshima and Barreras-Serrano (2002) studied the Pit-1 gene polymorphism in Baja California Holstein cattle where AA genotype had significant effect on milk yield as was reported by Renaville *et al.* (1997) in which Allele A to be linked with higher milk yield, more protein yield and less fat percentage.

Characterization of Pit-1 gene

The Summary of nucleotide variations corresponding to exon 6 of Pit-1 gene with respect to 451 bp fragment of Pit-1 gene presented in Table 5 respectively. No variations were noticed between Murrah, crossbred and zebu cattle. However one nucleotide variation have been noticed in wild yak when compared to Murrah buffalo and it was found in exon 6, occuring at 114th (G→C)) position. On comparison of Murrah buffalo with sheep and goat, six nucleotide variations were observed in the exon 6 which were at 115th (C→A), 177th (G→A), 198th (G→A), 223rd (A→C), 228th (A→C) and 243rd (C→T). On comparison of Murrah buffalo with *Sus scrofa*, fourteen nucleotide variations were observed which were 54th (T→A), 57th (A→G), 60th (C→T), 93rd (G→A), 105th (G→A), 112th (C→T), 115th (C→A), 132th (A→G), 150th (G→A), 168th (T→C), 198th (G→A), 201th (A→G), 204th (C→T) and 243th (C→T) positions.

The estimates of genetic relatedness (similarity and divergence) for the nucleotide and amino acid have been presented in Fig 5. The nucleotide sequence homology of Murrah was found to be highest (99.8%) with *Bos indicus* and *Bos taurus*, while it was lowest with *Sus scrofa* (89.5%). Analysis of deduced amino acid revealed that exon 6 coded for 69 amino acids (Fig 6). Only *Capra hircus* and *Ovis aries* had one amino acid change when compared to Murrah buffalo which was at 60th (Threonine→Proline) position. The deduced amino acid sequence showed 100% homology with *Bos indicus* and Bos taurus, *Bos mutus* and *Sus scrofa* and lesser similarity was noticed with *Capra hircus* and *Ovis aries* (98.6%).

Table 5: Summary of nucleotide variations corresponding to exon 6 of Pit-1 gene

	Exon 6 (From 44 to 252)																	
Nucleotide Position #	**54**	**57**	**60**	**93**	**105**	**112**	**114**	**115**	**132**	**150**	**168**	**177**	**198**	**201**	**204**	**223**	**228**	**243**
Buffalo (Murrah)	T	A	C	G	G	C	G	C	A	G	T	G	G	A	C	A	A	C
Zebu cattle	T	A	C	G	G	C	G	C	A	G	T	G	G	A	C	A	A	C
Crossbred cattle	T	A	C	G	G	C	G	C	A	G	T	G	G	A	C	A	A	C
Wild Yak	T	A	C	G	G	C	**C**	C	A	G	T	G	G	A	C	A	A	C
Sheep	T	A	C	G	G	C	G	**A**	A	G	T	**A**	**A**	A	C	**C**	**C**	**T**
Goat	T	A	C	G	G	C	G	**A**	A	G	T	**A**	**A**	A	C	**C**	**C**	**T**
Pig	**A**	**G**	**T**	**A**	**A**	**T**	G	**A**	**G**	**A**	**C**	G	**A**	**G**	**T**	A	A	**T**

Nucleotide position as per Buffalo sequence i. e. Consensus sequence

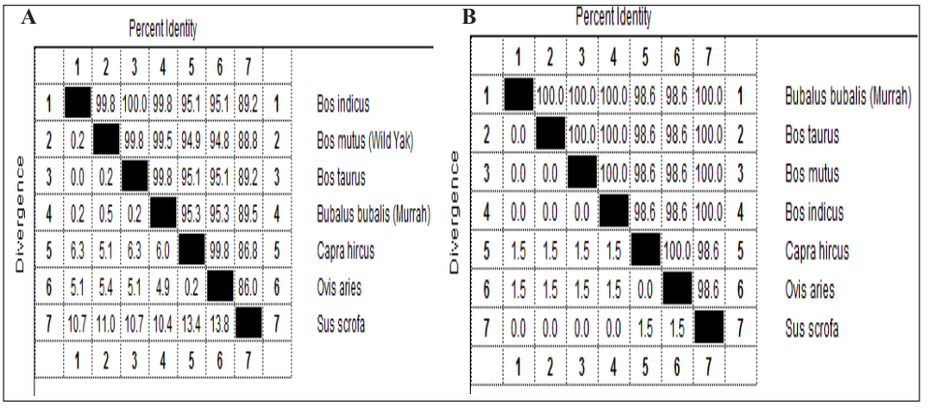

Fig. 5: Sequence homology on the basis of nucleotide (451bp) and Amino Acid (69bp) sequences of exon6 for Pit-1 gene

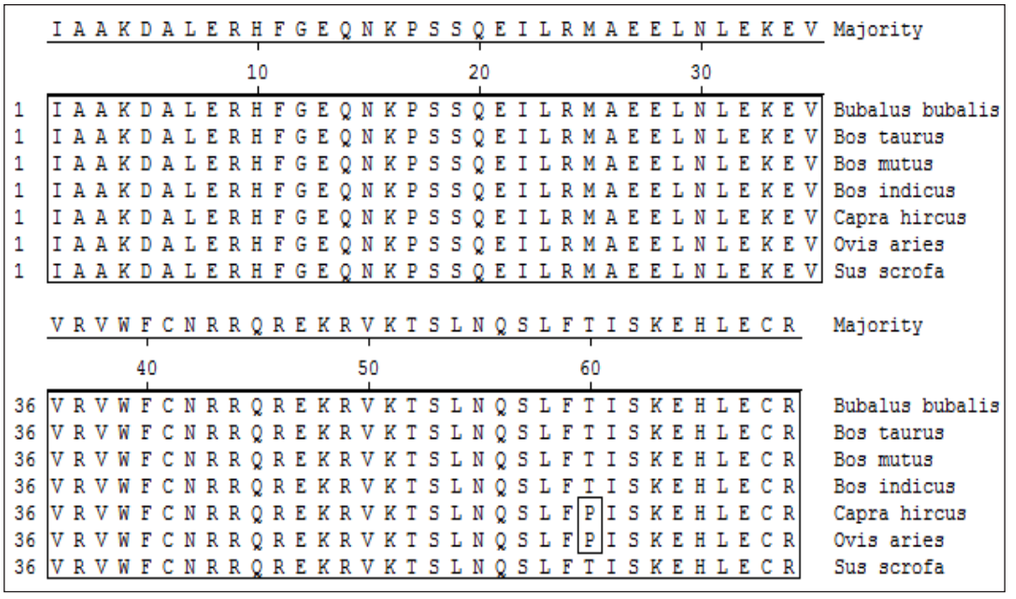

Fig. 6: Comparative analysis of amino acid sequence of Pit-1 gene (69bp)

It was concluded from these observations that PRL and Pit-1 genes were found to be monomorphic. The monomorphic pattern of exon 3 and exon 6 of both genes with respect to different enzymes indicate their conservedness within the herd. Sequence comparison of both genes of Murrah buffalo was also found to be closer to swamp buffalo than other ruminants for PRL gene and was closest to *bos species* for this amplicon. Further, if any polymorphism in these genes is identified in future studies, then association of these polymorphs with economic traits could be used as a genetic marker for selection programme.

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