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Allele fixation in pituitary-specific transcription factor 1 (Pit-1) Gene of Murrah Buffalo

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Abstract
The present investigation undertaken to study the genetic variability in the Pituitary specific transcription factor-1 gene's polymorphism and its association with performance traits was carried out using Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) and nucleotide sequencing techniques. DNA was isolated from 50 Murrah buffalo maintained at LUVAS, Hisar. A 600 bp fragment of the Pit-1 gene was amplified and digested using *Hinf*I restriction enzymes. The genotypes were then sequenced using the Sanger di-deoxy chain termination method and were compared with related species. *Hinf*I locus showed only one genotype. The amplicon of the gene exhibited the absence of polymorphism with respect to restriction enzymes used; accordingly, the allelic frequency was found to be unity. The monomorphic pattern of the amplicon of these genes concerning different enzymes indicated the concernedness of these genes. The result suggested that the allele has been fixed in the herd and needs the inclusion of the Murrah buffalo from other populations to introduce desired genetic variability to enhance milk production.

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

Introduction
The buffalo is considered a crucial milch animal in India, as more than half of the total milk produced is buffalo milk. The average milk productivity of buffaloes in India is much higher than indigenous cattle, which reveals the importance of buffalo compared to indigenous/nondescript cattle. In domestic animals, cattle, sheep and goats, the Pit-1 gene was located on chromosome 1 at the centromeric region (1q21-22) and identified *Hinf*1 polymorphism of bovine Pit-1 gene by RFLP method in exon 6 (Woollard *et al.*, 2000) [18]. Sequence analysis showed that the buffalo Pit-1 protein shares high homology with cattle - Bos Taurus (100%), goat (99%), sheep (99%), human (96%) and pig (98%) (Parikh and Rank, 2013) [14]. Ozdemir (2012) [13], while working with Holstein and Native Ear cattle in Turkey and Trakovicka *et al.* (2015), while working with Holstein cattle in the Slovak Republic, found polymorphism with Pit-1 gene digested by *Hinf*1 restriction enzyme. Chauhan *et al.* (2015) [3] in Sahiwal cattle using PCR-RFLP revealed 600 bp product and restriction digestion with *Hinf*1 showed three genotypes. Further, they discovered a significant difference among the three genotypes for total milk yield and milk yield at 300 days, with the AA genotype showing a higher value than AB and BB genotypes in the first lactation. Zabeel *et al.* (2018) [19], in native cattle in Kerbala City, found significant associations between Pit-1 gene polymorphism and milk yield traits. The same variants present different effects in different breeds due to different genomic background influences (Mattos *et al.*, 2004). Studies on Pit-1 gene polymorphism have been primarily on exotic cattle, but scanty information is available on the Murrah breed of buffalo. Thus, it is crucial to understand the genetic structure of potential genetic markers and their correlation with various performance traits in Murrah buffalo.

Materials and Methods
Genetic stock: The study was conducted on 50 Murrah buffalo maintained at Buffalo Research Center (Department of Livestock Production Management), Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, respectively. The relevant data of targeted performance traits under study were collected (Standard lactation milk yield and Lactation peak milk yield) from history cum pedigree sheets.

Blood Collection: Five ml blood in EDTA as an anticoagulant was collected in sterile vacutainers from the jugular vein collected, immediately placed in a collection box for transportation with coolant packs and brought to the Animal Genomics Laboratory,

Department of Animal Genetics and Breeding, College of Veterinary Sciences, LUVAS, Hisar. The blood samples were kept at -20 °C till the initiation of DNA isolation.

Extraction of Genomic DNA: Genomic DNA was isolated from the whole blood by Maxwell® RSC Whole Blood DNA Isolation kit. Cartridge Preparation: Gloves were worn before handling the kit, instruments, sample, reagents, and other plastic wear. Cartridges were placed in deck tray(s) with #1 well (the largest well in the cartridge) facing away from the elution tubes and pressed down on cartridge to snap it into position. The seal of the cartridge was carefully peeled back so that all plastic came off the top. Removal of all sealing tape and any residual adhesive was ensured. 50 µl of each blood sample was transferred from the starting blood tube to well #1 of each cartridge. The blood sample in well #1 was tip-mixed to ensure all blood had been transferred by using a different sterile pipette tip for every sample. One plunger was placed into well #8 (at the opposite end of well#1) of each cartridge. An empty 0.5 ml elution tube (provided in the kit) was placed into elution tube position for each cartridge in the deck tray. 60 µl of Elution Buffer was added to the bottom of each elution tube. The cartridge has then proceeded to Maxwell® Instrument Setup and Run.

Evaluation of Quality: The quality of the isolated genomic DNA was checked in horizontal submarine agarose gel electrophoresis using 0.6% w/v agarose Figure 1. Agarose gel electrophoresis. 10 ml TBE (10 X) + 90 ml double distilled water was measured by measuring cylinder, and Agarose powder (0.6g) was weighed in the electronic weighing balance and mixed in Flask, then heated in a microwave oven by heating it for four minutes with four interruptions (three times of one minute and two times for half minutes) and allowed to cool to lukewarm temperature. One µl of Ethidium bromide (10 mg/ml stock made to a working solution of 0.5µg/ml concentration) was added and mixed thoroughly. Cellophane tape was stuck on the open sides of the tray to form the mould. The melted agarose was poured into a gel fixer casting tray fitted with an acrylic comb (combs with various wells like 10, 14 and 20 were used as required) and allowed to solidify for 30 minutes. 2ul DNA samples were loaded into the wells and mixed with 6X loading dye. Among wells, one control well was loaded with 6X loading dye only. Electrophoresis was run at 5-6 volts/cm till the loading dye (Bromophenol blue) reached the other end of the gel. The DNA was visualised with a gel documentation system (BioradGeldoc EZ Imager). DNA samples showing sharp and intact bands were used further.

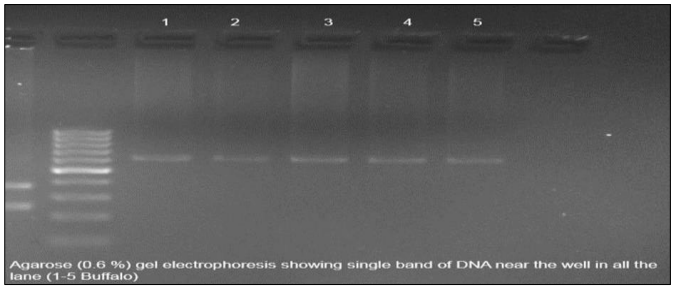

Fig 1: The quality of the isolated genomic DNA was checked in horizontal submarine agarose gel electrophoresis using 0.6% w/v agarose

Evaluation of concentration and purity
The concentration of genomic DNA was determined with the help of a UV spectrophotometer (Trekstar Surf Tab) by using the following formula

DNA concentration (µg DNA/m1) = $(\text{Optical Density})_{260}$ x 50 x dilution factor

The purity of DNA was checked by taking the ratio of optical density (OD) varying from 260 nm to that 280 nm. The samples with an OD ratio (260/280) between 1.7 and 1.9 were used in subsequent analysis.

Amplification by PCR
The target region of these genes was amplified by PCR. PCR protocols for denaturation, primer annealing and extension were standardised.

Composition of PCR Mixture
Genomic DNA - 2 µl (50 ng/µl); Forward primer - 0.5 µl (10 pM/µl); Reverse primer - 0.5 µl (10 pM/µl); Master mix - 12.5 µl; Nuclease free water - 9.5 µl; Total volume - 25 µl.

Primer sequence
For Gene Pit -1 Exon 6 SEQUENCES F: GAG CCT ACA TGA GAC AAG CAT C and R: AAA TGT ACA ATG TGC CTT CTG A producing a product size of 600 bp (Chauhan 2015) [3] procured from Sigma-Aldrich were used to amplify the target region, i.e. exon 6 of Pit-1 gene. Pit-1 F and Pit-1 R primers procured from Sigma were resuspensions of dried DNA oligos in 538 µl and 585 µl MQ water, respectively, to make 100 pm/ul stock solutions. Stock solutions were stored at -20 °C temperature. While the working solution was prepared by mixing 10 µl stock solution with 90 µl DNA-RNA free water, used for further PCR amplification process kept at 4 °C for short-term use. PCR amplification was done with BioRad

PCR-RFLP gene polymorphism of Pit-1 gene using Restriction enzyme *Hinf*1
Restriction fragment length polymorphism analysis is one of the most effective tools for monitoring animal genetic diversity. The gene's polymorphism was studied using restriction enzyme *Hinf*1 (Source: *Haemophilus influenzae* Rf) (Gene Loci at Pit -1 Intron 5 exon 6 having the Recognition Sites 5'-G ANTC-3'and 3'-CTNA G-5') as reported in the literature. 10 ul of amplified product was digested with 5 units of *Hinf*1 Enzyme overnight (for 14 hours) at 37 ° C in a water bath.

Composition of RE digest Optimised RE digestion mixture (Pit-1)
MQ water - 7.5 µl; 10 x buffer H for RE - 2 µl; Restriction enzyme *Hinf*1 - 0.5 µl (10U/µl); PCR product - 10 µl; Total volume obtained is 20 µl. The digested products were further electrophoresed in 2.5% agarose gets in 1 X TBE buffer. The ethidium bromide (10mg/ul) was added during agarose gel preparation. The gel was visualised under a gel documentation instrument Bio-RAD. The *Hinf*1 PCR-RFLP assay was produced with genotypes, as presented in Figure 2.

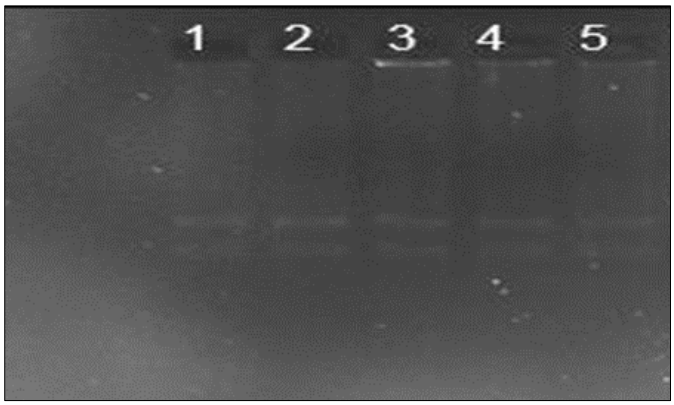

Fig 2: Agarose gel electrophoresis showing different bands of DNA near the well in all the lane

Nucleotide sequence analysis

Respective samples of identified genotypes from the sample population were sequenced. Sequences obtained were further analysed by using "Clustal W", an online bioinformatic computer program for multiple sequence alignment, and "Chromas 266", an offline bioinformatic computer program for SNP identification. Figure 3.

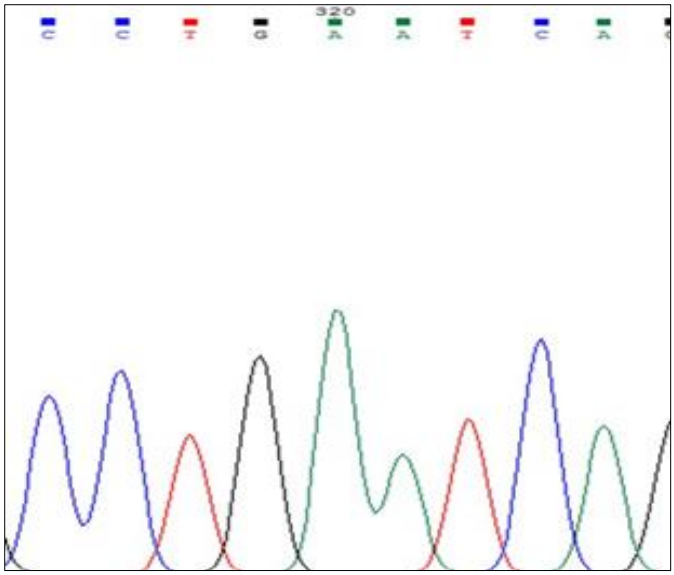

Fig 3: Portions of different sequences files obtained while reading Gene Sequences by using Chromas showing Murrah Buffalo GG

Estimation of gene and genotype frequencies: Gene and genotype frequencies were estimated by Falconer and Mackay (1996) [8] method.

Results

Calculation of allelic and genotypic frequency: The allelic and genotypic frequencies were calculated and presented in Table 1. The genotypic frequencies from the studied sample size of Murrah buffalo of 50 sample size were calculated by the following method. Genotypic frequency = total no. of individuals of a particular genotype divided by the total no of individuals taken for study for the same gene.

Genotype frequency (AA) = 0/50 = 0.0000
Genotype frequency (AB) = 0/50 = 0.0000
Genotype frequency (BB) = 50/50 = 1.0000

Allelic frequency is the total no. of alleles of the particular type of allele (i.e. the sum of heterozygous and twice homozygous of that allele) divided by the total number of alleles for that locus in the population (i.e. twice the number of individuals understudy)

Allelic frequency (A) = (2D+H)/2N = (2*0+0)/(2*50) = 0/100 = 0.0000

Allelic frequency (B) = (2R+H)/2N = (2*50+0)/(2*50) = 100/100= 1.0000

On analysis of the table 1 found that the proportion of B allele was found high in the population as compared with A

Table 1: The allelic and genotypic frequencies were calculated and presented

Total no. of Obs.	Genotypes			Allele Frequency	
	AA	BA	BB	A	B
50			1 (50)		1

Discussion

Allelic frequency is the total number of alleles of the particular type of allele (i.e., the sum of heterozygous and twice homozygous of that allele) divided by the total number of alleles for that locus in the population (i.e., twice the number of individuals understudy). Results revealed that in Murrah buffaloes, the allelic frequency for A and B was obtained 0.0000 and 1.0000; the buffaloes used for the present study were monomorphic. Pit-1 polymorphism was associated with cattle's milk yield and conformation traits (Renaville *et al.*, 1997) [15]. Doosti *et al.* (2011) [7] reported that AA genotypes of the Pit-1/*Hinf*I genotype could be helpful in fertility and create the next generation for an increase in milk production and growth of Holstein cattle. Cosier *et al.* (2012) reported polymorphism about significant milk yield, fat and protein per cent and confirmed this locus as the candidate gene that may produce differences in milk characteristics and can be used in marker-assisted selection in Romanian Simmental cattle. Aggarwal *et al.* (2010) [1] also reported the monomorphic Pit-1 genetic makeup in Murrah buffaloes. However, Ahmadzadeh *et al.* (2019) [2] reported polymorphic findings for the Pit-1 gene in Khuzestan (Iran) water buffaloes. Furthermore, in Murrah buffalo (Mavi *et al.*, 2017) [10], Egyptian buffaloes (Othman *et al.*, 2011) [12] and Indonesia buffaloes (Misrianti *et al.*, 2010) [11] reports of Pit-1 allele fixation was reported; however, a study in Mehsana Buffalo (Parik and rank, 2013) [14] reported polymorphism. Association studies on larger buffalo population having different breeds involving larger number of candidate genes should be conducted to exploit possible genetic variability potential in selection procedures to enhance the rate of genetic improvement.

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