MOLECULAR CHARACTERIZATION OF THE GROWTH HORMONE GENE IN RIVERINE BUFFALOES

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ABSTRACT

Growth hormone, a protein hormone, has a remarkable role in growth, reproduction and milk production. The present investigation was aimed at studying the genetic variation in growth hormone gene locus 4th exon, 4th intron and 5th exon in Murrah buffalo bulls by PCR-RFLP. The growth hormone gene was amplified by PCR using oligonucleotide primers standardized for *Bos taurus* species. A 428 bp fragment of growth hormone gene spanning over 4th exon, 4th intron and 5th exon was amplified and digested with *Alu* I restriction enzyme using a cattle specific primer. The sizes of the amplification products were similar in cattle and buffalo. Growth hormone gene in buffalo reveals monomorphism since no variation was found. The result indicates strong conservation of DNA sequence between cattle and buffalo. Nucleotide sequence variations and amino acid variation were observed at growth hormone gene between *Bubalus bubalis* and *Bos taurus* species reveals 97.1% nucleotide identity 12 nucleotide substitutions, but a single amino acid change. A phylogenetic tree constructed for buffalo with other species revealed buffalo were closer to cattle and yak compared to other species. The growth, milk production, seminal and sexual behavioral parameters could not be associated with buffalo growth hormone genotypes due to their monomorphic haplotype.

Keywords: growth hormone gene, PCR-RFLP, genetic polymorphism, phylogenetic tree, haplotype

INTRODUCTION

Growth hormone is a protein hormone consisting of 191 amino acids. Reports suggest that growth hormone has remarkable role in growth, reproduction and milk production. Growth hormone helps in body growth and metabolism through protein synthesis, protein deposition in tissues and organs (Gluckman *et al.,* 1987), increased nitrogen retention (Hart and Johnson, 1986), gluconeogenesis and cell division (Neathery *et al.,* 1991), intestinal calcium absorption, thereby enhancing overall bone growth and stimulating chondrocyte proliferation, (Boyd and Baumann, 1989). It has been found that growth hormone increases the metabolic priority of the mammary gland (Elvinger *et al.,* 1988; Binelli *et al.,* 1995). Growth hormone augments testosterone action in the maintenance of spermatogenesis (Boccabella *et al.,* 1963) and acts on sertoli cells releasing IGF-I, which then acts on the leydig cells of the testis and

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releases testosterone in mice (Ritzen, 1983; Waites *et al.,* 1985). In the case of AI bulls, a positive relationship between GH and IGF-I concentrations and semen quality has been observed, with a higher (3.2 times) level of GH in seminal plasma than in blood plasma (Davis *et al.,* 1985).

The growth hormone gene has been assigned to the 19q17 position of bovine chromosome (Hediger *et al.,* 1990). Bovine growth hormone gene consists of five exons and four introns (Gordon *et al.,* 1983). Genetic polymorphism at growth hormone gene mainly arises from several point mutations at the gene. Polymorphism studies of bovine growth hormone gene have been conducted on the coding (Lechniak *et al.,* 1999), intron (Hoj *et al.,* 1993) and promoter regions (Rodrigues *et al.,* 1999) of the gene.

The buffalo contributes about 54 percent of the total milk produced in India. Although the economic importance of buffaloes has always been known, yet very little work has been carried out to exploit the genetic potential of this animal. Though studies have been carried out on characterization in cattle, similar studies in buffaloes are scarce.

The reports regarding polymorphism studies in buffaloes are very scanty, so the present investigation was carried to find the polymorphism of growth hormone gene in buffalo and to associate such polymorphic pattern with growth traits, milk production traits, seminal and sexual behavioral traits, needed during semen collection and evaluation in artificial breeding programme for genetic improvement of buffalo.

MATERIALS AND METHODS

Animals

The present study was conducted with 130 Murrah buffalo bulls maintained at Artificial Breeding Complex of the National Dairy Research Institute, Karnal, Haryana, India, and Frozen Semen bull station, Salboni, Paschim Medinipur, West Bengal and other bull breeding stations in West Bengal, India.

Sample and data

About 10 ml venous blood was collected from the jugular vein of each animal in a sterile 50 ml polypropylene vial containing 0.5 M EDTA as anticoagulant.

Data on birth weight, three-month body weight, six month body weight, expected predicted difference (EPD) and superiority (percent) over the herd average of the bulls were collected from the records maintained.

The data on behavioral traits such as libido score, reaction time, Flehmen's response, requirement of mounting stimulus and various seminal traits like semen volume, sperm mass activity, seminal consistency and individual fresh sperm motility, post thaw sperm motility and number of semen doses per collection were collected during daily semen collection and evaluation. The ejaculates were collected twice a week. For all practical purposes the freezing of semen was done with the mass activity more than 3.5 and the lower standard for sperm concentration remain $20x10^6$ motile sperm per semen dose. The bulls exhibiting Flehmen's response were coded as 1.0 and 2.0 when no response was obtained. Likewise when external stimulus was needed for mounting, the code was 1.0 and when no stimulus was needed, the code was 2.0. Libido was scored on a 10-point scale (Chenoweth, 1976). Reaction time was measured as the time taken by a bull from its introduction to female (a dummy in the present study) until first ejaculation, and it is an important

indication of male sexual behaviour. The bulls exhibiting Flehmen's response were coded as 1.0 and 2.0 when no response was obtained. Likewise when external stimulus (as whistling sound) was needed for mounting, the code was 1.0 and when no stimulus was needed the code was 2.0.

DNA preparation

Genomic DNA was isolated from blood samples following the phenol- chloroform extraction method described by Sambrook and Russel (2001).

DNA was dissolved in TE buffer and was kept in a water bath at 60°C for 2 h to dissolve pellet properly in buffer. The quality of DNA was checked through spectrophotometry. DNA samples with O.D. ratio between 1.7 and 1.9 were considered as good and used for further study. The samples beyond this range were re-extracted by the phenol-chloroform extraction method. DNA quality was also checked by running the sample in 0.8 percent agarose gel electrophoresis. The DNA samples devoid of smear were used for further study.

DNA amplification

A 428 bp fragment of the growth hormone gene spanning over 4th exon, 4th intron and 5th exon was amplified with forward (5′CCGTGTCTATGAGAAGC3′) and reverse (5′GTTCTTGAGCAGCGCGT3′) primer sequences. PCR was carried out in a final volume of 25 µl reaction mixture containing 80-100 ng DNA, 2.5 µl 10X PCR assay buffer, 200 mM of each dNTP, Taq DNA polymerase, 20 pmM of each primer and 2 mM $MgCl_2$. To check contamination, a negative control, labelled 'C' with master mix devoid of template DNA was made. PCR-reactions were carried out in a thermocycler (PTC-200, MJ Research, USA) in two stages. In the first stage, initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 46°C for 45 seconds, extension at 72°C for 50 seconds were carried out for 10 cycles. In the next stage, denaturation at 94°C for 1 minute, and annealing at 48°C for 45 seconds were carried out, followed by final extension at 72°C for 50 seconds for the next 25 cycles.

RFLP and polyacrylamide gel electrophoresis

The 428 bp amplicon was digested with Alu I enzyme to identify polymorphism of growth hormone gene. Amplified product was digested with 10 U Alu I restriction enzyme at 37°C for overnight and finally, reaction was stopped after adding 0.5 m EDTA.

The digested product was separated through 8% non-denaturing polyacrylamide gel run at 100V for 5 h. The gel was stained with silver nitrate, as described by Bassam *et al.* (1991), with slight modifications where 10% glacial acetic acid was used for fixing the DNA bands for 30-45 minutes, before staining with 0.1% silver nitrate for 30 minutes. The developer used was 3% sodium carbonate containing 300 µl of formalin. After staining, the fragments were visualized and documented in gel doc system.

Nucleotide sequencing

The PCR products were concentrated to 50 ng/µl by pooling several tubes to precipitate by the isopropanol procedure. In order to obtain clean fragment for sequencing, the PCR products were separated by electrophoresis in a TAE agarose gel containing ethidium bromide using standard protocols. The desired PCR product band was excised using a clean, sterile razor blade or scalpel (band was visualized in a medium or long

wavelength (e.g., ≥300 nm) UV light, and excised quickly to minimize exposure of the DNA to UV light). The minimum agarose slice was transferred to a 1.5 ml microcentrifuge or screw cap tube and then purified by using commercially available gel extraction kits (Qiagen). Quantification was done by loading one µl of eluted sample in 1% agarose gel and comparing with standard molecular marker (Phi X 174 DNA ladder or 100 bp DNA ladder). Only samples with good concentration (>50 ng/µl) were selected and subjected to sequencing.

Sequence data analysis

Sequence data were analyzed mostly by DNASTAR software.

Database search

The database search of sequences for a possible match to the DNA sequence of growth hormone gene was conducted using the BLAST algorithm available at the National Center for Biotechnology Information (NCBI, Bethesda, MD). Translated protein sequences of different growth hormone genes were also subjected to the BLAST algorithm.

Statistical analysis

The frequencies of gene and genotypes were estimated for the identified locus as per the method suggested by Falconer and Mackay (1996). Association study with the growth, milk production, seminal and sexual behavioral parameters with the genotype of growth hormone gene could not be achieved due to monomorphism of the alleles of growth hormone gene.

RESULTS AND DISCUSSION

Identification of Genotypes

The PCR amplification generated a 428 bp segment from growth hormone gene which is homologous to the cattle growth hormone gene of similar length (Figure 1), thus it indicates strong conservation of DNA sequences in both species. A single restriction pattern was observed in all the samples of Murrah buffalo bulls producing four bands consisting of 265 bp, 96 bp, 51 bp and 16 bp (Figure 2) and was assigned as the LL genotype corresponding to leucine homozygote for cattle. Thus, all the buffalo bulls were found to be monomorphic, which was in agreement with the report of Biswas *et al*. (2003) , who observed 70 Murrah, 32 Bhadawari, 30 Jaffarabadi, 30 Surti and 30 Nagpuri and found only the LL genotype with *Alu*I PCR-RFLP. The present study is also in agreement with the studies conducted by Mitra *et al*. (1995). Aravindakshan *et al*. (1997) also reported monomorphism of growth hormone gene at 3rd intron of growth hormone gene using MspI as restriction enzyme. Therefore, this monomorphism of the buffalo may be a species specific characteristic of buffalo. Thus, the gene and genotypic frequencies were found to be 1.00.

Nucleotide sequencing

Monomorphism of the genotype of growth hormone gene was confirmed by nucleotide sequencing. The nucleotide sequence as well as the derived amino acid sequence of growth hormone gene of riverine buffalo (Gene Bank Accesion number GU223914) have been depicted in Figure 3.

Nucleotide sequence variations and amino acid variation were observed at the growth hormone gene between *Bubalus bubalis* and *Bos taurus*

Table 1. Mean±S.E. of growth traits of Murrah bulls.

Birth Weight (kg)	3 M Body Weight (kg)	6 M Body Weight (kg)
33.83±0.95	62.53±2.24	100.88±3.64

Table 2. Mean±S.E. of behavioural characteristics Murrah bulls.

Libido Score (Score)	Reaction Time (Second)	Flehmen's Response (Score)	Mounting Stimulus (Score)
5.07±0.46	43.13±11.36	1.33±0.09	1.25±0.10

Table 3. Percent of behavioural and seminal characteristics shown Murrah bulls.

Flehmen's Response (%)	Mounting Stimulus (%)	Seminal Consistency (%)
65.00	72.22	50.00[a] 40.00[b] 10.00[c] –[d]

a = Creamy; b = Lemon; c = Milky; d = Watery.

Table 4. Mean±S.E. of seminal characteristics of Murrah bulls.

Semen Volume (ml)	Mass Activity (0-5)	Individual Motility (%)	Individual Motility (Score)	Post-Thaw Motility (%)	Post-Thaw Motility (Score)	Semen Doses per Collection (No.)
2.70±0.48	2.10±0.13	58.40±4.15	3.18±0.23	44.98±1.63	2.80±0.10	133.59±9.98

Table 5. Mean±S.E. of production traits of Murrah.

EPD (kg)	Superiority (%)
242.52±26.14	11.03±1.20

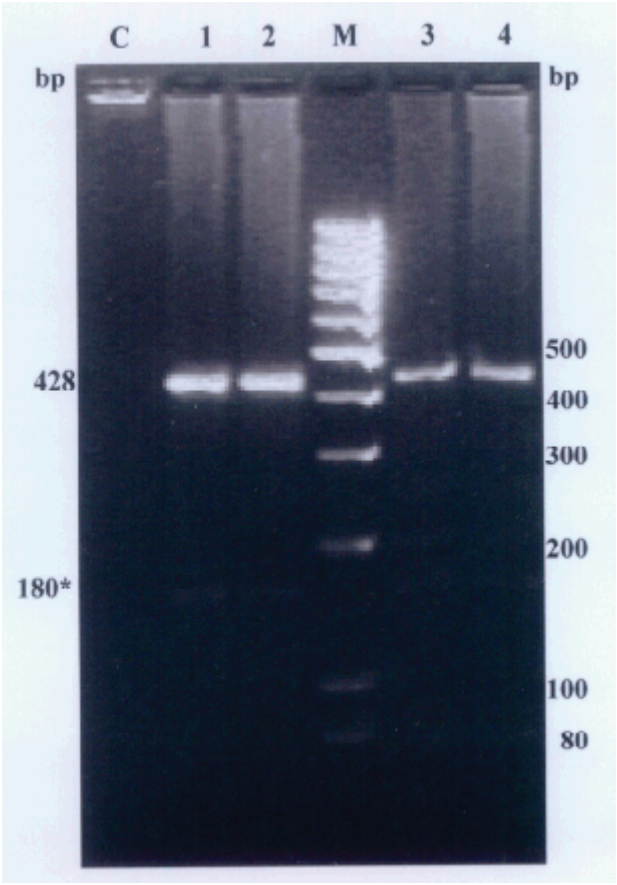

Figure 1. PCR product of buffalo and cattle growth hormone gene in agarose gel electrophoresis.
Lane C represents control.
Lane 1, 2 represents PCR product obtained from cattle bulls.
Lane 3, 4 represents PCR product obtained from Murrah buffalo bulls.

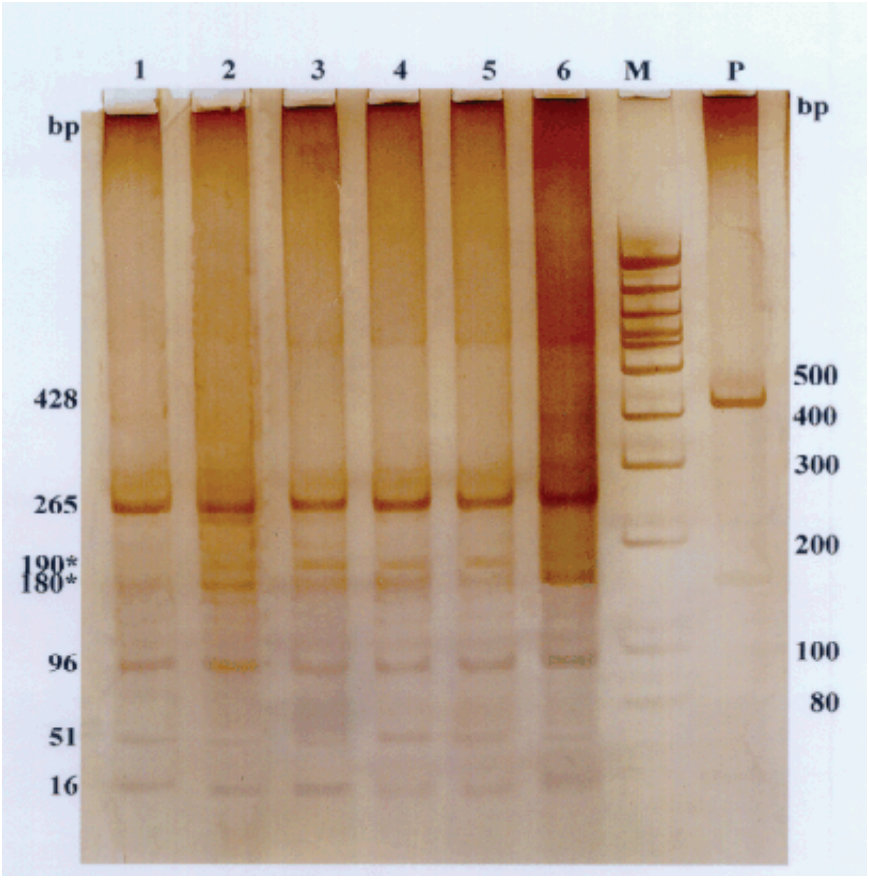

Figure 2. PCR-RFLP of 428 bp fragment of growth hormone gene in buffalo bulls.

Lane P : 428 bp PCR product.

Lane M: 100bp marker.

Lane 1-6 : LL genotype.

* indicates the presence of non-specific bands.

Nucleotide sequence:

CTGAAGGACCTGGAGGAAGGCATCCTGGCCCTGATGCGGGTGGGGATGGCGTTGTGGGTCCC
TTCCATGCTGGGGGCCAT
GCCCGCCCTCTCCTGGCTTAGCCAGGAGAACGCACGTGGGCTGGGGGAGAGAGATCCCTGCT
CTCTCACTCTTCCTAGCA
GTCCAGCCTTGACCCACCCCAAACCTTTTCCCCTTTTGAAACCTCCTTCCTCGCCCTTCTCCAA
GCCTGTAAGGGAGGGT
GGAACATGGAGCGGGCAGGAGGGAGCTGCTCCTGAGGGCCCTTCGGCTCTCTGTCTCTCCCTC
CCTTGGCAGGAGCTGGA
AGATGGCACCCCCCGGGCTGGGCAGATCCTCAAGCAGACCTATGACAAATTTGACACAAACAT
GCGCAGTGACGACGCGC
TGCTCAAGAAC

Amino acid sequence:

LKDLEEGILALMRELEDGTPRAGQILKQTYDKFDTNMRSDDALLKN

Figure 3. Sequence report :Amplified 411 bp fragment of growth hormone gene exon 4, intron4 and exon5 in *Bubalus bubalis* breeds (Gene bank acc no. GU223914).

Pair Distances of ALL SPP CORRECTED.meg J. Hein (Weighted).

Percent Similarity in upper triangle.

Percent Divergence in lower triangle.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	
1	***	**97.1**	47.5	47.5	99.5	40.2	92.3	46.9	76.3	67.4	95.1	47.2	43.4	57.6	46.9	81.5	45.3	95.2	YAK
2	3.0	***	**47.3**	**47.3**	**97.1**	**40.3**	**91.7**	**46.8**	**75.2**	**66.2**	**93.9**	**47.0**	**43.3**	**57.2**	**46.8**	**80.7**	**45.2**	**94.2**	BUFFALO
3	92.8	93.3	***	92.0	47.7	76.4	48.9	90.2	49.9	54.2	23.1	94.7	78.9	46.1	88.2	49.7	87.7	46.9	CAMEL
4	92.4	92.9	8.5	***	47.7	76.4	48.7	95.5	49.9	53.1	23.1	92.5	78.9	44.6	88.2	50.3	86.0	46.4	CAT
5	0.5	3.0	91.5	91.1	***	40.5	92.3	47.2	75.8	67.1	95.1	47.5	43.7	57.9	47.2	81.5	45.6	95.2	CATTLE
6	100.0	100.0	28.5	28.4	100.0	***	42.1	76.2	43.9	46.3	17.6	76.4	67.4	43.0	74.7	41.7	74.7	40.2	CHICKEN
7	8.2	8.8	87.1	87.5	8.2	100.0	***	48.7	75.8	65.2	91.5	48.7	43.7	56.8	48.4	82.7	46.8	91.8	DEER
8	94.6	95.1	10.6	4.7	93.3	28.7	87.5	***	47.8	51.8	22.6	92.0	77.9	44.6	86.7	49.0	85.0	46.9	DOG
9	29.3	30.7	83.9	83.5	30.1	100.0	29.9	91.0	***	67.0	75.6	50.9	47.0	57.6	47.8	76.0	48.3	74.7	DONKEY
10	43.8	45.9	70.9	73.6	44.3	94.4	48.2	77.3	44.9	***	71.1	55.0	47.6	56.6	50.8	64.3	51.0	65.2	ELEPHANT
11	81.6	82.1	9.7	11.7	80.6	28.4	76.9	12.9	80.5	70.5	***	44.7	34.2	70.4	39.2	79.3	22.1	97.1	GOAT
12	94.2	94.7	5.5	8.0	92.8	28.4	88.3	8.5	80.7	68.8	9.7	***	79.2	46.4	88.7	49.7	89.2	47.2	HORSE
13	100.0	100.0	25.2	25.2	100.0	43.3	100.0	26.7	95.1	90.9	29.4	24.8	***	48.4	75.4	44.3	74.9	43.2	HUMAN
14	64.0	64.8	96.7	100.0	63.4	100.0	65.4	100.0	65.7	66.8	91.7	95.0	87.7	***	44.3	57.2	44.3	56.5	MONKEY
15	94.6	95.1	13.0	12.9	93.3	31.1	88.8	14.9	91.0	80.5	15.1	12.4	30.8	100.0	***	46.9	96.5	45.8	MOUSE
16	21.6	22.6	84.2	82.3	21.6	100.0	19.8	86.8	29.4	49.8	83.1	84.2	100.0	65.6	94.1	***	46.4	80.5	PIG
17	100.0	100.0	13.6	15.7	99.5	31.2	94.4	17.1	88.6	79.6	17.4	11.8	31.6	100.0	3.6	95.6	***	44.8	RAT
18	5.1	6.1	94.8	96.6	5.0	100.0	8.8	94.4	31.7	48.2	81.8	93.9	100.0	66.5	99.0	23.0	100.0	***	SHEEP
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	

Figure 4. Genetic similarity of buffalo with other species with respect to growth hormone gene.

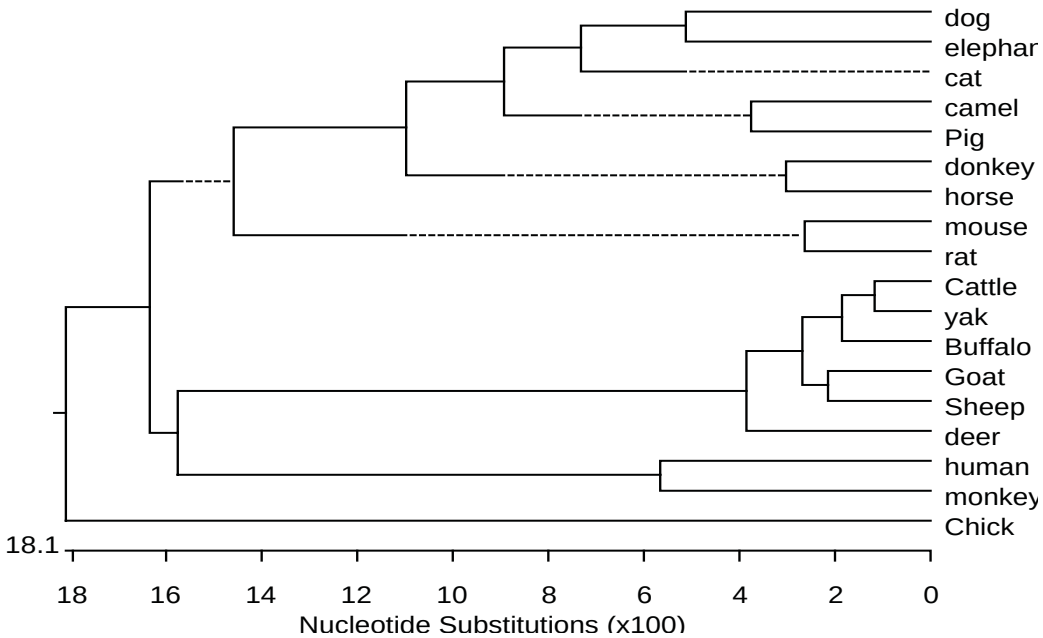

Figure 5. Phylogenetic analysis of buffalo with other species with respect to growth hormone gene.

species reveals 97.1% nucleotide identity. Comparison of the growth hormone gene sequence of cattle and buffalo, pertaining to 4th exon, 4th intron and 5th exon revealed 12 nucleotide substitutions, but a single amino acid change (Figure 4). The remaining three codons were synonymous. The nucleotide sequence organization is similar to other species, as goat, sheep, mouse, human, monkey, camel, cat, chicken, deer, dog, donkey, elephant, horse, yak, pig and rat (www. ncbi.nlm. nic.in)

Phylogenetic study of buffalo with other species with respect to growth hormone gene

The phylogenetic tree for buffalo with other species has been depicted in Figure 5. The phylogenetic tree constructed for buffalo with other species revealed the buffalo was closer to cattle and yak compared to other species (Figure 5), thus bovines were grouped together. Buffalo were genetically closer to goat and sheep. This pattern was similar to the phylogenetic tree constructed based on the nucleotide sequence of CD14 gene from different species (Pal and Chatterjee, 2009). Deer was also clustered with the above mentioned species, indicating ruminants were grouped together. Buffalo was found to be genetically most distant to chicken, thus depicting completely different lineage.

The average value of the phenotypic parameters including growth traits (Table 1), reproductive traits (Tables 2, 3, 4) and production traits (Table 5) have been depicted. The growth, milk production, seminal and sexual behavioral parameters could not be associated with buffalo growth hormone genotypes due to their monomorphic haplotype.

CONCLUSION

Bovine growth hormone gene specific primers amplified the buffalo growth hormone and PCR amplification yielded a 428 bp fragment spanning from 4th exon, 4th intron and 5th exon from buffalo DNA homologous to that of cattle. AluI restriction enzyme yields 265 bp, 96 bp, 51 bp and 16 bp fragments. Based on available information in cattle, all the buffalo DNA samples were genotyped as LL. Thus the monomorphic pattern of growth hormone gene in buffaloes may be a species specific characteristics of buffalo.

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