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Genetic polymorphism of leptin gene in relation with production traits in Murrah buffaloes

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Abstract
Leptin is one of the gene involved in immune functions, fertility and regulation of feed intake. In the present study 50 lactating Murrah buffaloes were screened to identify the polymorphism in exon-2 and exon-3 of leptin gene through PCR-RFLP by using 2 restriction enzymes *Msp*I and *Hin*d III. *Msp*I had revealed two fragments in exon-2 (210bp, 79bp) and exon-3(255 bp, 150 bp). Whereas, Hind *III* did not reveal any cutting site in both the exons. PCR-RFLP patterns in the exon-2 and exon-3 with the two restriction enzymes employed revealed the monomorphic nature of the leptin gene so the production traits were not correlated with polymorphism as no allelic variants were found in the gene. The overall least squares mean for total lactation milk yield, standard lactation milk yield, lactation length, peak yield, attainment of peak yield were 1,943.77 ± 84.56 kg, 2008.27 ± 82.15kg, 291.13 ± 9.23 days, 11.88 ± 1.36 kg and 25.40 ± 1.28 days, respectively.

Keywords: Leptin gene, polymorphism, PCR-RFLP

Introduction
Leptin, a 16kD protein that is synthesized by adipose tissue is involved in regulation of feed intake, fertility, energy balance and immune functions. The leptin gene consists of two introns and three exons which spans about 18.9kb. The size of exon-2 is 289bp and exon-3 is 405bp which are separated by intron of approximately 2 kb. The present study was undertaken with the objective to identify polymorphisms in leptin gene and its association with milk production traits (Total lactation milk yield, standardized lactation milk yield, lactation length, peak yield, attainment of peak yield) in Murrah buffalo.

Materials and Methods
Blood samples were collected from 50 lactating Murrah buffaloes (Bubalus bubalis) maintained at Livestock Research Station, Mamnoor of P V Narasimha Rao Telangana Veterinary University. The DNA was isolated from blood through a standard phenol chloroform method. Quality of the DNA was checked by running the sample in 0.8% agarose gel and quantity by spectrophotometry.

Table 1: Description of primers used and the amplified product of different loci

Region	Primer sequence 5'-3'	Primer length bp	Amplified product
Exon-2	F-5' - GGT GGT AAC GGA TCA CAT GG -3' R-5' - CCA CGG TTC TAC CTC GTC TC -3'	20 20	289
Exon-3	F-5' - GCA TAG CAG TCC GTC TCC TC -3' R-5' - TTC CCT GGA CTT TGG GAA G -3'	20 19	405

F: Forward, R: Reversed

A master mix for PCR amplification was prepared as presented in the Table 2

Table 2: Composition of PCR reaction mix for amplification

S. No	Components	Volume	Final concentration
1	10x Taq buffer	1.25 µl	1 X
2	dNTPs (10mM)	0.5 µl	0.4 mM
3	Primer-Forward (50 pM)	1 µl	10 pM
4	Primer-Reverse (50 pM)	1 µl	10 pM
5	MgCl2(25 mM)	0.5 µl	1 mM
6	Taq Polymerase(1unit/µl)	0.5 µl	0.5 unit
7	Autoclaved Mille Q water	6.75 µl	_

An aliquot of 11.5 µl of master mix per sample was drawn into thin-walled PCR tubes and 1 µl (50-100 ng) of template DNA was added for making 12.5 µl of PCR mix. The PCR tubes were marked for the identification, then spinned briefly for proper mixing and mounted in PCR machine.

Table 3: PCR reaction conditions for exon -2 region of leptin gene

Step	Process	Temperature (°C)	Time
1	Initial denaturation	94	5 min
2	Cyclic denaturation	94	1 min
3	Primer annealing	53	35 sec
4	Cyclic extension	72	40 sec
5	Steps 2 to 4 were repeated for 30 cycles		
6	Final extension	72	10min
7	Hold	4	Forever

Table 4: PCR reaction conditions for exon-3 region of leptin gene

Step	Process	Temperature (°C)	Time
1	Initial denaturation	94	5 min
2	Cyclic denaturation	94	1min
3	Primer annealing	44	35sec
4	Cyclic extension	72	30 sec
5	Steps 2 to 4 were repeated for 35 cycles		
6	Final extension	72	5 min
7	Hold	4	Forever

The amplified products were confirmed by visualizing on 1.5% agarose under UV transilluminator and documented by gel documentation system.

Restriction enzyme digestion of PCR products (RFLP): The PCR products of exon-2 and exon-3 of leptin gene was digested with *Msp*I and *Hind*III restriction enzymes at 37°C for overnight in total volume of 20µl reaction mix containing, restriction enzyme (10U/ µl) 0.2 µl, restriction buffer 2 µl, nuclease free water 7.8µl were applied on the exon-2 with 10 µl of PCR product each time. The digested products were run on a 2% agarose gel and visualized on gel doc system for analysis.

Results and Discussion

Amplification of PCR product generated a 289 bp for exon-2 (Fig.1) and 405 bp for exon-3 segment (Fig. 2) for leptin gene of buffalo. Amplified PCR products were subjected to enzyme digestion.

The *Msp*I digestion of exon-2 resulted in two fragments of size 210 bp and 79 bp (Fig.3) and exon-3 resulted two fragments of size 255 bp and 150 bp (Fig.4) where as *Hind*III did not reveal any cutting site in exon-2 (Fig.5) and exon-3 (Fig.6) in all 50 samples. Hence the leptin gene showed monomorphic band pattern revealing no allelic variants.

Similar findings were observed by Manisha *et al*. (2014) [6] in Mehasana buffaloes by *Hph*I restriction enzyme. Datta *et al.* (2012) and Sanjoy *et al*. (2013) [1, 7] reported monomorphic nature of leptin gene in Murrah buffalo. Srikala *et al*. (2006) [8] by using *Hae*III, *Hph*I, *Sau*3AI, *Alu*I and Kale *et al.* (2013) [3] by *Bsa*AI, *Sau*3AI *and Kpn*2I restriction enzymes in water buffaloes also observed monomorphic pattern of the gene. It might be because of the fact that the allele must be conserved as the animals were in closed herd.

Whereas Jamuna *et al*. (2016) [2], reported three types of genotypes (CC, TC, TT) in the exon-2 of leptin gene in Murrah buffaloes by using *Aci*I restriction enzyme confirming its polymorphic nature. Kumar *et al*. (2018) [5] by *hph*I in Sahiwal cattle observed polymorphism of leptin gene and reported two genotypes CT, TT for exon-2.

In the present study, as there were no allelic variants observed in leptin gene for two restriction enzymes, correlation of the leptin gene with production traits could not be established. However, data related to milk production traits of the animals under study were analyzed by using univariate GLM model to obtained the least square means. The overall least squares mean for total lactation milk yield, standard lactation milk yield, lactation length, peak yield, attainment of peak yield were 1,943.77 ± 84.56 kg, 2008.27 ± 82.15kg, 291.13 ± 9.23 days, 11.88 ± 1.36 kg and 25.40 ± 1.28 days, respectively. The least squares mean obtained for all the production traits under study did not reveal any significant effect. This might be due to sampling error, differences in the age of animals, genetic constitution of the herd and managemental practices of the farm. However, the studies of Thiruvenkadan *et al*., (2014) and Kumar *et al*., (2017) [9, 4] in Murrah buffaloes showed significant effect ($P< 0.05$) of parity on all the production parameters.

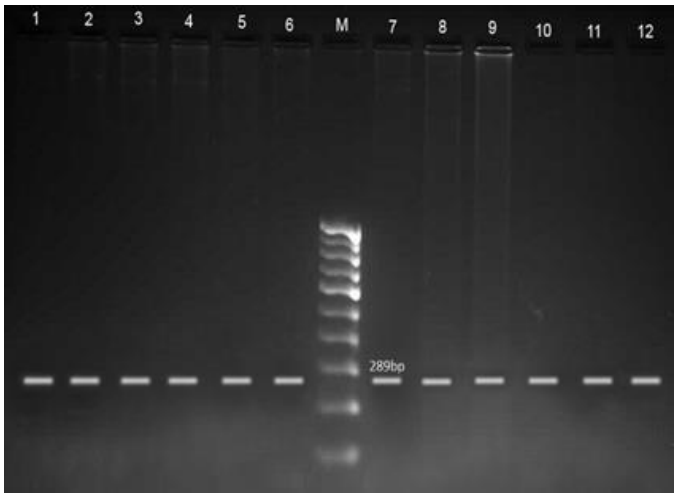

Fig 1: Amplified PCR product of exon-2 (289bp) on 1.5% agarose gel
Lanes 1-6 and 7-12: PCR product (289 bp) Lane M: 100bp Molecular Marker

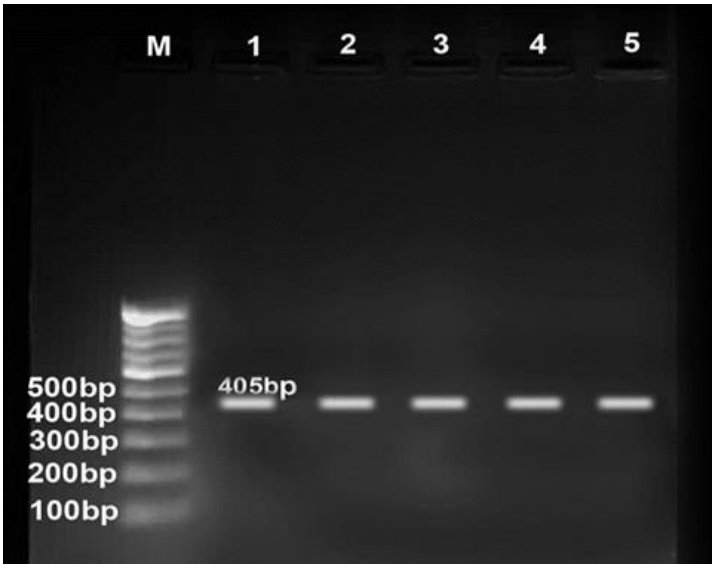

Fig 2: Amplified PCR product of exon-3 (405 bp) on 1.5% agarose gel
Lanes 1-5-PCR product (405 bp) Lane M: 100 bp Molecular Marker

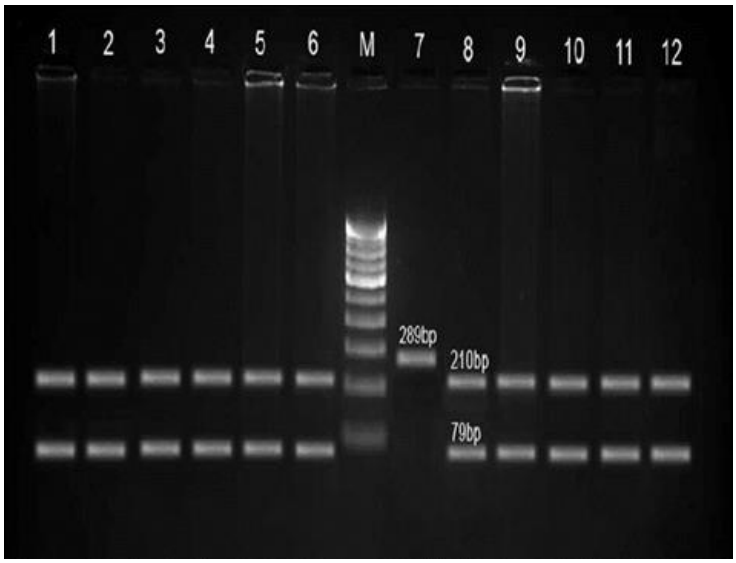

Fig 3: PCR-RFLP of exon-2 of Leptin gene on 2.5% agarose gel using *Msp*I RE Lanes 1-6 and 8-12: 2 Bands (210 bp and 79 bp)
Lane7: PCR product (289 bp) Lane M: 100 bp Molecular Marker

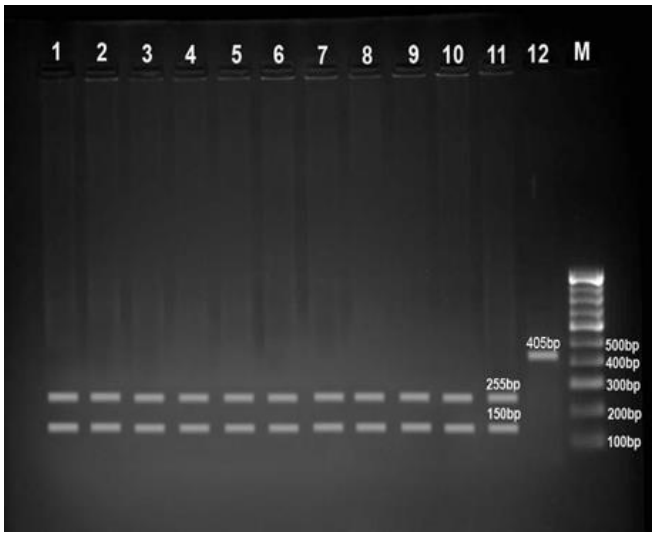

Fig 4: PCR-RFLP of exon-3 of Leptin gene on 2.5% agarose gel using *Msp*I RE Lanes 1-11 Bands (255 bp and 150 bp) Lane12: PCR product (405 bp)
Lane M: 100 bp Molecular Marker

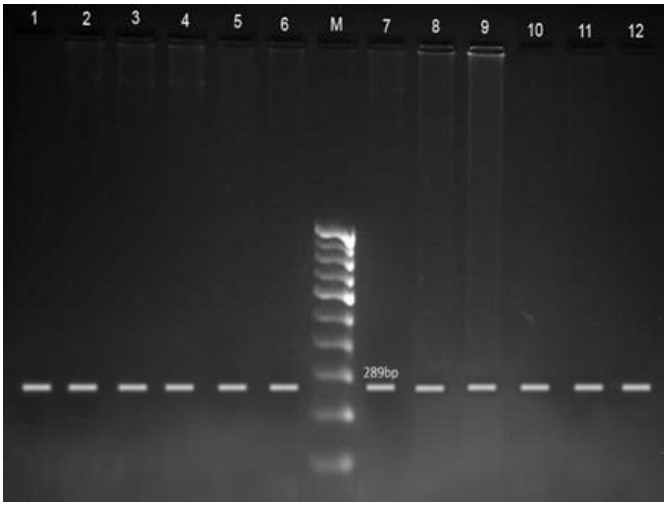

Fig 5: PCR-RFLP of exon-2 of Leptin gene on 2.5% agarose gel using *Hin*dIII RE Lanes 1-6 and 8-12: uncut PCR product (289bp)
Lane 7: PCR Product (289 bp) Lane M: 100 bp Molecular Marker

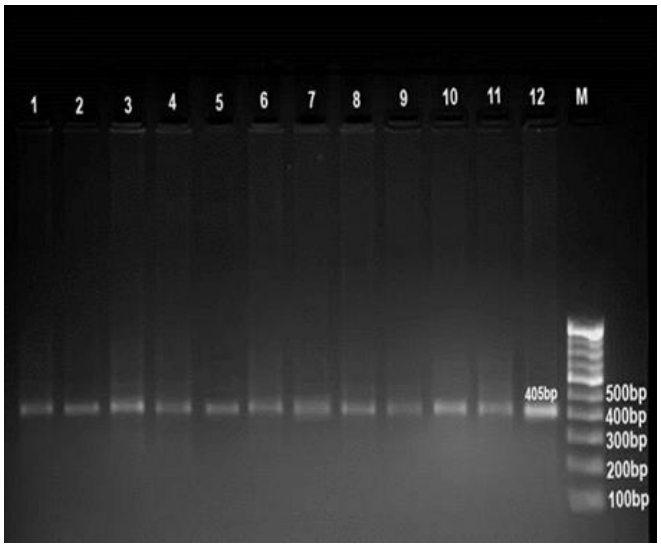

Fig 6: PCR-RFLP of exon-3 of Leptin gene on 2.5% agarose gel using *Hin*dIII RE Lanes 1-11: uncut PCR product (405 bp) Lane12: PCR Product (405 bp)
Lane M: 100 bp Molecular Marker

Table 5: Least squares analysis of variance of production traits

Source of Variation	d.f.	TLMY		SLMY		LL		PY		APY	
		M.S.S	E.S.S	M.S. S	E.S.S	M.S. S	E.S.S	M.S. S	E.S.S	M.S. S	E.S.S
Parity	6	444742.93	202902.68	288124.25	191484.2	3734.13	2420.07	46.37	52.62	93.09	46.97

Significance at $P \leq 0.05$

Table 6: Least squares means of production traits

Overall mean	TLMY			SLMY			LL			PY			APY		
	n	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.
	50	1,943.77	84.56	50	2,008.27	82.15	50	291.13	9.236	50	11.88	1.362	50	25.401	1.287
Parity															
1	8	1,774.75	159.25	8	1,804.06	154.71	8	297.25	17.39	8	9.36	2.56	8	22.50	2.42
2	2	2,417.50	318.51	2	2,404.50	309.42	2	308.00	34.78	2	9.75	5.12	2	22.50	4.84
3	15	1,523.47	116.30	15	1,716.37	112.98	15	255.60	12.70	15	15.10	1.87	15	27.73	1.77
4	11	1,875.05	135.81	11	1,867.88	131.93	11	309.45	14.83	11	13.14	2.18	11	24.18	2.06
5	3	1,768.72	260.06	3	1,839.70	252.64		285.67	28.40	3	11.00	4.18	3	20.00	3.95
6	9	1,890.45	150.14	9	2,001.11	145.86	9	293.44	16.39	9	15.33	2.41	9	30.89	2.28
7	2	2,356.50	318.51	2	2,424.27	309.42	2	288.50	34.78	2	9.50	5.12	2	30.00	4.84

Means followed by same superscripts do not differ significantly ($P \leq 0.05$)

Summary

Leptin gene was amplified with specific primers and PCR amplification yielded amplicon of exon-2 and exon-3 of bubaline leptin gene. All Murrah buffaloes included in present study are monomorphic as revealed by PCR-RFLP analysis using *Msp*I, *Hind*III restriction enzymes. Thus, the monomorphic pattern of leptin gene in buffaloes may be a species-specific characteristic of buffalo. There was no allelic variants observed for leptin gene in the present study so correlation of the gene with the production traits could not be established.

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