Int.J.Curr.Microbiol.App.Sci (2020) *9*(6): 1514-1524

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 9 Number 6 (2020)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2020.906.187

Assessment of Genetic Variability in Prolactin Gene and Its Impact on Milk Composition in *Bos indicus* Cattle

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Keywords

Prolactin, RFLP, Sequence polymorphism, Milk composition

Article Info

Accepted:
18 May 2020
Available Online:
10 June 2020

A B S T R A C T

The candidate gene effect of prolactin gene (*PRL*) on milk traits suggests the important role of genetic variability in prolactin gene on milk composition parameters. Therefore the present study was conducted to explore the genetic variation in *PRL* gene through sequence based RFLP marker approach and to observe their association with milk composition in three *Bos indicus* cattle breeds (Rathi, Sahiwal and Kankrej). Genomic DNA was extracted from blood samples collected from 225 randomly selected milking cows maintained at Livestock Research Stations at Bikaner. A 156 bp fragment length of *PRL* gene was amplified through species specific primer. Milk samples from respective animals were analyzed on three different occasions for estimation of different constituents of milk. RFLP marker detected the presence of two genotypes; *GG* and *AG* in each breed. Sequence analysis identified an overall four nucleotide substitutions found to be responsible for genetic variation in *PRL* gene. Association analysis revealed the presence of significant association between genetic variability in prolactin gene and milk protein content with no impact on other milk constituents. The present study concluded that heterozygotes genotypes for *PRL* exon-3 region were favored in studied *Bos indicus* breeds to produce milk containing higher amount of protein.

Introduction

India is homeland and origin place of many elite cattle breeds of the world and possesses around 40 well recognized *Bos indicus* breeds of cattle (Anonymous, 2013) which holds great promise and potential for milk production in addition to their exemplary adaptability to tropical climate. The effective assessment of genetic variability in genes affecting the physiological pathways of milk composition traits (Agrawal *et al.*, 2018) and incorporation of this genotypic information as fixed factor in estimation of breeding value could significantly improve the selection responses and potential values of the indigenous cattle genetic resources (Mohammed *et al.*, 2015). Newer breeding goals especially on milk composition traits have recently gained importance following

the demands of a healthier human diet (Krovvidi *et al*., 2013).

The assessment of genetic variability in genes affecting milk traits in indigenous cattle could establish their association with lactation performance (Agrawal *et al.*, 2017). The candidate gene approach (El-Magd *et al*., 2014) have detected bovine prolactin (b*PRL*) gene as one of the potential candidate gene that have significant impact on milk composition traits in dairy cattle (Komisarek and Dorynek, 2009) due to their positional role on chromosome 23 at 43 cM, close to the quantitative trait loci region (QTLs) of 36, 41, and 42 cM (Bennewitz *et al*., 2004). The prolactin gene invariably affects growth and development of mammary gland (mammogenesis), synthesis of milk (lactogenesis) and maintenance of milk secretion (galactopoiesis) (Dong *et al*., 2013). The indirect effect of prolactin gene on milk production traits occurs through its modulatory role on thermoregulatory mechanisms during hot climates (Alamar, 2011) and regulatory role in calcium homeostasis through enhanced intestinal calcium absorption (Charoenphandhu and Krishnamra, 2007). In addition, transcript product of prolactin gene is known to initiate a signaling cascade that activates the transcription of a number of genes, including expression of milk protein, lactose and lipid genes (Othman *et al*., 2011) via Janus kinases/signal transducers and activators of transcription (JAK STATs) signal transduction pathway (Hu *et al*., 2009) through binding with prolactin receptor gene (*PRLR*).

As most of the earlier studies have reported polymorphism within the *bPRL* gene on the basis of restriction fragment length polymorphism (RFLP) without explaining the nature and locations of nucleotide substitutions, the present investigation was planned to comprehensively detect the genetic variability in prolactin gene through sequence based RFLP marker approach and its impact on milk composition in three different *Bos indicus* cattle.

Materials and Methods

Selection of Animals: Apparently healthy and mastitis free 225 milking *Bos indicus* cows with 120 days lactation were selected at random from pure breeding population of Rathi (n=75), Sahiwal (n=75) and Kankrej (n=75) maintained at Livestock Research Stations of Rajasthan University of Veterinary and Animals Sciences, Bikaner (Rajasthan) under similar managemental and feeding conditions in a grid position of 28.02ºN and 73.31ºE with mean annual rainfall of 277.55 mm.

Extraction of Genomic DNA and PCR Amplification: Genomic DNA was extracted from 2 ml whole blood collected from jugular vein in an Ethylene Diamine Tetra Acetic acid (EDTA) containing sterile vacutainer tube through Genomic DNA Isolation Kit (Himedia Pvt Ltd) as per manufacturer's protocols. Nano-drop spectrophotometry and 0.8% agarose electrophoresis was carried out to assess the purity (OD ratio 260/280) and concentration (ng/µl) of extracted genomic DNA. A 156-bp fragment of exon-3 of *PRL* gene was amplified by polymerase chain reaction (PCR) in a final reaction volume of 25µl with reaction mixture of 5X PCR buffer (5µl), 1.5mM $MgCl_2$ (3µl), 10 Mm dNTP's mix (1µl), forward primer 70pmol/µl (1µl), reverse primer 70 pmol/µl (1µl), genomic DNA 25 ng/µl (4µl), Taq DNA polymerase 5U/µl (0.2µl) and DNAase free water (10.8µl). A set of common forward and reverse primer pair (F5'-CGAGTCCTTATGAGCTTGATTCTT-3'; R3'-GCCTTCCAGAAGTCGTTTGTTTTC-5') was constructed as suggested by Mitra *et*

al., (1995) for indigenous cattle. Amplification was performed in a thermal cycler with following program; after an initial denaturation step at 95°C for 5 min, 35 cycles were programmed as follows: 95°C for 45s, 59°C for 45s, 72 °C for 60s and final extension at 72°C for 10 min. The amplified DNA fragments were stained and visualized on 1.5% agarose gel under Gel Documentation System.

Assessment of Genetic variability in *bPRL* gene: The initial screening for polymorphism at specified nucleotide position was carried out through RFLP method. The 156 bp amplicons of exon-3 region of *PRL* gene was digested in reaction mixture containing 10X buffer (2µl), amplified product (10µl), restriction enzyme *Rsa* I 10 units (1µl) and nuclease free water (17µl) at 37ºC for 6 hr in water bath and electrophoresed on 8% polyacrylamide gel at 120V for the probable nucleotide substitution at GTAC recognition site. The gels were stained with 1% ethidium bromide solution and visualized with under UV light and documented by gel documentation system. The determination of prolactin genotypes was carried out on the basis of number and size of the digested fragments: (*GG* 156 bp), *AG* (156 bp, 82 bp, 74 bp) and *AA* (82bp, 74 bp) (Udina *et al*., 2001).

At least five representative PCR products under each electrophoresed RFLP patterns in each breed were purified through Exosap method and sequenced in both directions in an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) through Sanger dideoxy chain termination method for sequence analysis and SNP's detection. The raw sequences were analyzed with Chromas 1.45 (http://www.technelysium.co.au) software for the detection of sequencing anomaly and forward and reverse sequence of each gene fragment was assembled against the most closely related reference sequence through BLAST algorithms http://www.ncbi.nlm.nih.gov/BLAST) and Bioedit Sequence Alignment Editor (*ver.* 7.0.5.3) software. Multiple sequence alignment (MSA) for sequenced region of *PRL* gene was carried out through Clustal W software in order to detect the precise location of SNP's. Generated sequences were submitted to National Center for Biotechnology Information (NCBI) GenBank database for assignment of accession numbers.

Collection and Analysis of Milk Samples: About 100 ml was collected from each animal during mid milking stage in clean, dry, grease free and labeled milk collection vials on the same test day of each month for three consecutive months. Animals in terminal stage of lactation were not selected for milk collection. The collected milk samples were transported under ice to Milk Analysis Laboratory of Uttari Rajasthan Milk Union Limited (URMUL), Bikaner (Rajasthan) for the analysis of different milk constituents through Automated Milkoscan Tester (Bentley Instruments Inc., Chaska, USA).

Impact of Genetic Variability on Milk Composition Parameters: The differences among genotypes within breed were tested for significance of association with milk composition values using least square method of SPSS *ver.* 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical analysis was carried out through following general linear model (GLM):

$$X_{ij} = \mu + g_i + e_{ij}$$

where-

X_{ij} = mean observed value of milk performance parameter, µ= general mean

gi= fixed effect of the i^{th} genotype, e_{ij}= random error, $e_{ij} \sim NID\ (0, \sigma_e^2)$

Results and Discussion

An integrated approach through RFLP marker in conjunction with sequence analysis as suggested by Alipanah *et al*., (2007a) was adopted in the present study to detect the genetic variability which could be used as more informative marker for their impact on milk composition traits. The RFLP marker based assessment of genetic variability in 156-bp fragment of *PRL* gene at *A/G* SNP position revealed the presence of two genotypes, *GG* and *AG* with complete absence of genotypic pattern *AA* in all the studied *Bos indicus* cattle breeds (Fig. 1). The allele *A* was detected in heterozygous form only. The studied breeds revealed similar genetic structures in term of analyzed *A/G* SNP despite their different origins. Similar restriction patterns using *Rsa* I restriction enzyme were reported by Kumari *et al.*, (2008) in exotic and Zebu cattle, Sacraverty *et al.*, (2008) in Kankrej cattle and Ghasemi *et al.*, (2009) in Montebeliard cows. The genotypic frequency of *GG* genotype ranged from 0.19 to 0.36, whereas such frequency for *AG* genotypic pattern ranged from 0.64 to 0.81 (Table 1). The relatively higher percentage of heterozygotes observed in Sahiwal breed could be due to over exploitation of pure bred animals of this precious genetic resource (Anonymous, 2013). The *A* allele of prolactin exon-3 was found to be more prevalent in Sahiwal cattle (0.41) than Rathi (0.36) and Kankrej cattle (0.32).

Similar to present investigation, Aravindakshan *et al.*, (2004) in Vechur cattle and Kumari *et al.*, (2008) in different *Bos indicus* breeds reported higher frequencies of *AG* genotypes. An excess of heterozygotes (0.63) with nearly similar gene frequency of both allele was also reported in Sahiwal based crossbred cattle 'Frieswal' (Bukhari *et al*., 2013). The minor differences observed in allele frequencies among studied *Bos indicus* breeds is in contrast to significant breed differences observed by Sharifi *et al.*, (2010) in Najdi cattle and Alfonso *et al.*, (2012) in American Swiss cattle. The significant departure ($p<0.001$) in gene and genotypic frequency from Hardy Weinberg equilibrium for exon-3 of *PRL* gene as revealed through chi square statistics suggest the role of natural fitness in *Bos indicus* cattle for milk related traits (Mac-Hugh *et al*., 1997).

Table.1 Gene and genotypic frequencies of exon-3 of *PRL* gene detected through RFLP analysis

Breed	Genotypic frequencies			Allele frequencies		Std. Error	Chi^2	p value	Significance
	GG	AG	AA	G	A				
Rathi (75)	0.28 (21)	0.72 (54)	---	0.64	0.36	0.039	23.22	0.000	**
Sahiwal (75)	0.19 (14)	0.81 (61)	---	0.59	0.41	0.040	34.58	0.000	**
Kankrej (75)	0.36 (27)	0.64 (48)	---	0.68	0.32	0.038	16.20	0.000	**
Overall	0.28 (62)	0.72 (163)	---	0.64	0.36	0.023	72.06	0.000	**

Table.2 List of position and nature of SNP's observed in exon-3 of *PRL* gene

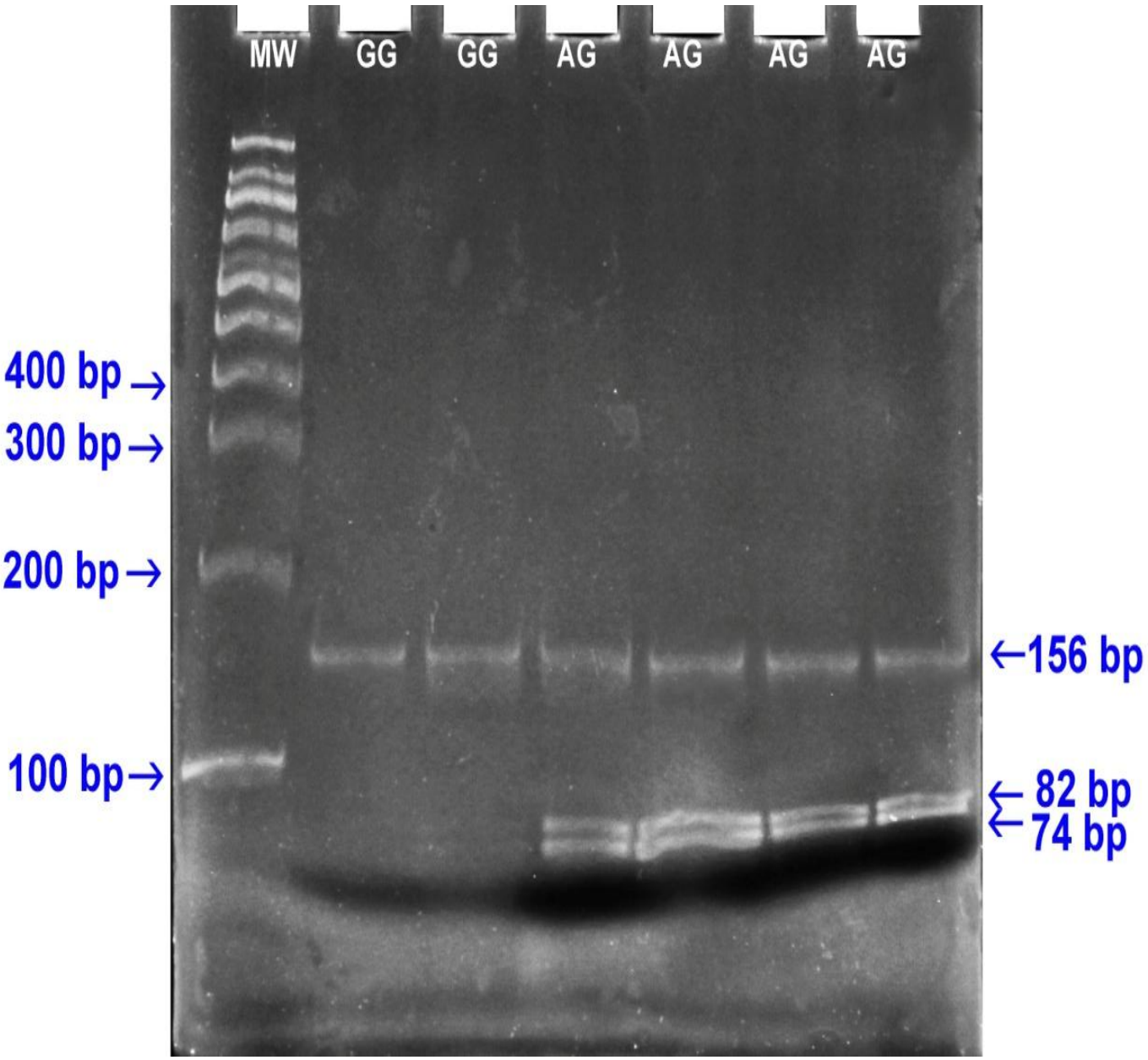
Base position	Region	Change	Nature of SNP's
39	Coding	C>T	Transition
58	Coding	C>T	Transition
59	Coding	C>A	Transversion
75	Coding	A>G	Transition

Table.3 Effect of genotypic patterns of exon-3 of *PRL* gene on milk composition parameters

Breed	Genotype		P- value
	GG	AG	
Fat (%)			
Rathi (75)	4.47±0.17	4.55±0.11	0.661
Sahiwal (75)	4.43±0.12	4.37±0.06	0.669
Kankrej (75)	4.50±0.12	4.44±0.09	0.865
Over all (225)	4.46±0.08	4.45±0.05	0.945
Protein contents (%)			
Rathi (75)	2.78^{a}±0.05	2.93^{b}± 0.03	0.022
Sahiwal (75)	2.98^{a}±0.10	3.23^{b}±0.05	0.027
Kankrej (75)	3.09^{a}±0.04	3.21^{b}±0.03	0.023
Over all (225)	2.96^{a}±0.04	3.12^{b}±0.03	0.001
Lactose (%)			
Rathi (75)	4.75±0.16	4.66±0.10	0.647
Sahiwal (75)	4.95±0.12	4.73±0.06	0.113
Kankrej (75)	4.81±0.04	4.79±0.03	0.623
Over all (225)	4.82±0.07	4.72±0.04	0.207
Solid not fat (%)			
Rathi (75)	8.42±0.07	8.51±0.04	0.282
Sahiwal (75)	8.80±0.10	8.77±0.05	0.824
Kankrej (75)	8.80±0.05	8.70±0.04	0.080
Over all (225)	8.67±0.04	8.66±0.03	0.846
Total solids (%)			
Rathi (75)	16.61±0.52	16.67±0.33	0.924
Sahiwal (75)	16.23±0.34	16.15±0.16	0.839
Kankrej (75)	16.37±0.21	16.15±0.16	0.400
Over all (225)	16.64±0.17	16.50±0.10	0.479

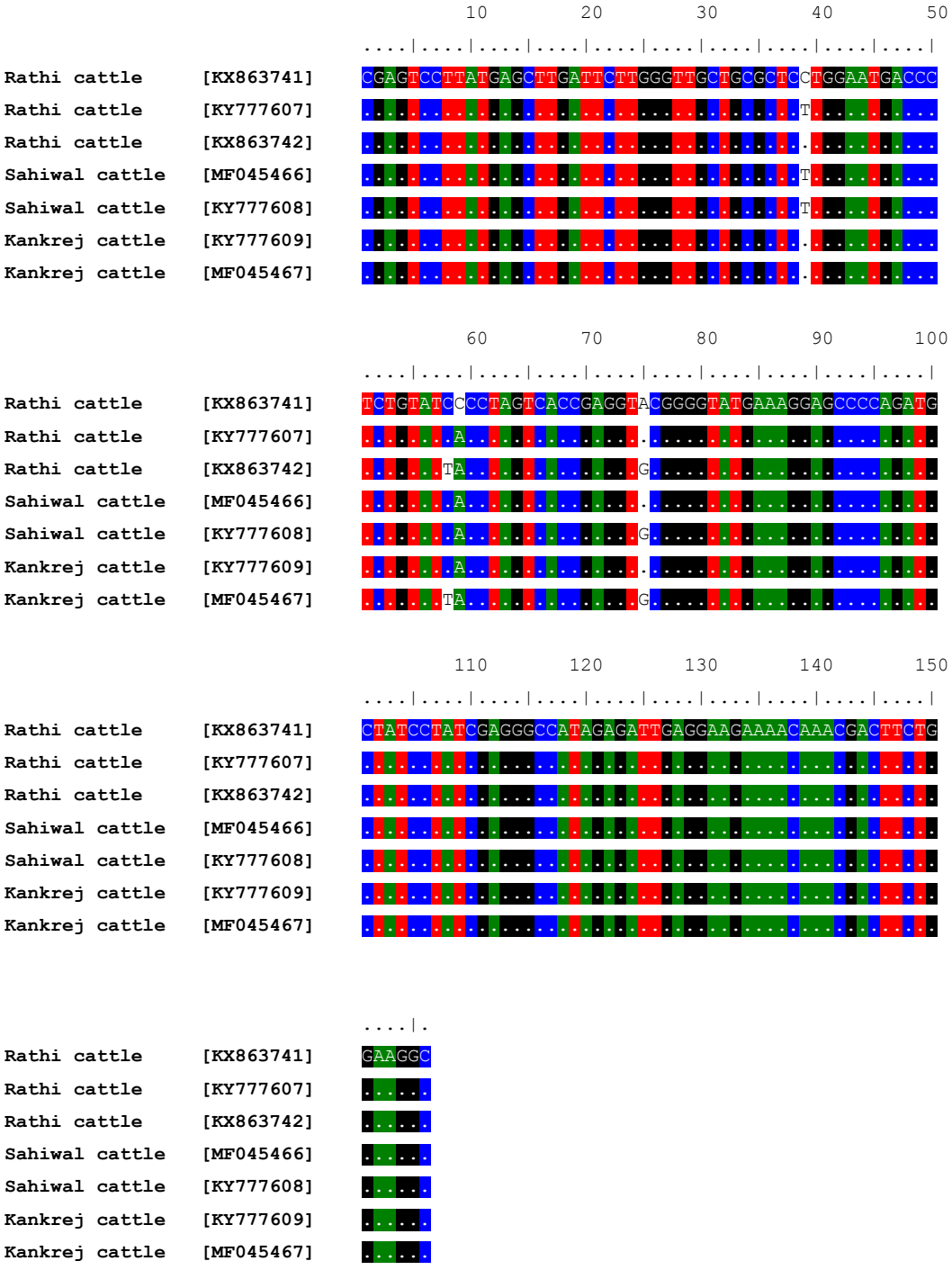
Means in the same row bearing different superscripts are significantly different ($p<0.05$).
NS Non-significant

Fig.1 Restriction fragment analysis of exon-3 of *PRL* gene



Lane 1: Molecular weight marker, Lane 2-3: genotype *GG*, Lane 4-7: genotype *AG*

Fig.2 Multiple sequence alignment of exon-3 of *PRL* gene



Similar absence of Hardy-Weinberg equilibrium was reported in two naturally adapted native Turkish breeds (Unal *et al.*, 2015) and in American Swiss cattle (Alfonso *et al.*, 2012). However, in contrast Ghasemi *et al.*, (2009), Sharifi *et al.*, (2010) observed non significant changes in gene and genotypic frequency in Montebeliard and Najdi cattle of

Iran, respectively which could be due to breed differences or reduced number of analyzed samples (n<50), leading to poor and inefficient representation of different genotypes (Brym *et al*., 2005).Similar absence of Hardy-Weinberg equilibrium was reported in two naturally adapted native Turkish breeds (Unal *et al*., 2015) and in American Swiss cattle (Alfonso *et al.,* 2012). However, in contrast Ghasemi *et al.,* (2009), Sharifi *et al.,* (2010) observed non significant changes in gene and genotypic frequency in Montebeliard and Najdi cattle of Iran, respectively which could be due to breed differences or reduced number of analyzed samples (n<50), leading to poor and inefficient representation of different genotypes (Brym *et al*., 2005).

The MSA and clustal analysis of accessioned sequences revealed that genetic variability contained in the different RFLP patterns of *PRL* gene in studied *Bos indicus* cattle were the result of substitution of four bases located at 39th, 58th, 59th and 75th position (Fig. 2). The SNP identified at 75th position was responsible for the occurrence of restriction site in exon-3 of *PRL* gene. The SNP's observed at 39th, 58th and 75th positions were transition in nature whereas SNP detected at 59th position was transverse mutation (Table 2). The current study reported less number of SNP's than Sasavage *et al.,* (1974) and Kaminski *et al.,* (2005) who identified seven and five SNP's, respectively in prolactin gene. The present study is in sharp agreement with the study conducted by Halabian *et al.,* (2008) which reported four SNP's in a similar fragment of 156-bp in Iranian Holsteins cows of which two SNP's were reported to alter the predicted sequence of amino acids.

Analysis of milk composition for the three studied breeds revealed a similar non-significant impact of *Rsa* I alleles of *PRL* exon-3 genes on milk fat in studied breeds (Table 3) as observed by Alipanah *et al.,* (2007b) in Red Pied cattle. In contrast, Chung and Kim (1997) reported higher milk fat for *GG* and *AG* genotypic pattern than *AA* genotype. Significantly varied values of milk protein were observed for the detected restricted pattern of exon-3 of *PRL* gene in all the studied *Bos indicus* breeds. The significantly higher milk protein detected for *AG* genotype in each breed and on overall basis do suggest the specific role of A allele in enhancement of milk protein. Non significantly higher milk lactose was detected in animals having *GG* genotype irrespective of the breed. The variation in SNF and total solid content of milk was also observed non-affected with the two detected genotypic patterns in any of the breed. Very few studies have investigated the effect of *PRL* genotypes on the milk composition traits. Similar to present investigation, results in Red Pied cattle showed that cows with *AA* genotype had higher protein yield and but lesser fat (p<0.05) (Alipanah *et al*., 2008).

In conclusion, the detection of several polymorphic sites in the nucleotide sequence of the coding region of exon 3 of bovine *PRL* gene in *Bos indicus* cattle in the present study create great possibility to use this genetic marker information for the genetic characterization of cattle populations for milk production traits and the sharing of polymorphism information on indigenous cattle genetic resources on national and global basis. The study concludes that heterozygotes genotypes for *PRL* exon-3 gene were favored to produce milk containing adequate amount of protein.

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How to cite this article:

Vijay Kumar Agrawal, Gyanchand Gahlot1, Rajeev Joshi, Govind Singh Dhakad and Sonal Thakur. 2020. Assessment of Genetic Variability in Prolactin Gene and Its Impact on Milk Composition in *Bos indicus* Cattle. *Int.J.Curr.Microbiol.App.Sci.* 9(06): 1514-1524.
doi: https://doi.org/10.20546/ijcmas.2020.906.187