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Research Article

GENETIC VARIATIONS IN THE MAPK 15 GENE AND THEIR RELATIONSHIP WITH MILK PRODUCTION CHARACTERISTICS IN MURRAH BUFFALO

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ABSTRACT: The current research explored the link between genetic diversity in the Mitogen-activated protein kinase 15 (MAPK 15) gene of buffalo and the traits related to milk composition and production, employing PCR-SSCP and Sanger sequencing techniques. The MAPK 15 gene, situated on bovine chromosome 14 and encompassing 1,892 base pairs, consists of 14 exons and encodes the enzyme Mitogen-activated protein kinase 15 (566 amino acids). This enzyme plays an essential role in the development of the mammary glands and the lactation process. It engages in intracellular signalling pathways that oversee various cellular functions, including growth and differentiation, suggesting its potential impact on milk production and the properties of milk composition. The modified high-salt approach was used to extract genomic DNA from blood samples taken from 100 Murrah buffaloes. PCR amplified products were resolved by 8% non-denaturing PAGE. Resolved PCR products were genotyped according to the band pattern during SSCP. The examination uncovered a uniform pattern during PCR-SSCP, hinting at a likely lack of variation, which implies a significant level of preservation in Murrah buffaloes. The uniform structure of the MAPK15 gene in this research can assist in distinguishing the Murrah breed from various other buffalo breeds. The candidate gene needs further validation in a larger cohort for use in the selection of buffalo cows with required milk composition traits.

Keywords: MAPK 15 gene, PCR-SSCP, Expression, Association, Milk composition traits, Murrah buffolo.

INTRODUCTION

The dairy business is the primary source of revenue for dairy farmers in India and is considered one of the country's economy's most significant and dynamic agrifood sectors. The commercial viability of dairy farming is intimately linked to dairy cows' milk yield and composition. Milk volume and fat concentration are two crucial variables that directly impact the profitability and effectiveness of dairy operations. India is the foremost milk producer globally, achieving a remarkable total dairy output of 239.3 million tonnes in 2024 [1]. Buffaloes represent nearly 50% of the cattle demographic (109.85 million) yet account for approximately 49.2%, while native cattle yield about 20% of the nation's overall milk output [2]. The Murrah buffalo is the most famous and widely recognized dairy breed globally, celebrated for its substantial milk production, high butterfat levels, and impressive growth potential [2, 3]. Murrah buffaloes typically yield about 2000 kg of milk over a lactation period of 305 day [3]. This variety enhances indigenous buffalo groups across different parts of Asia and other global regions.

Enhancing milk production and composition features in dairy cows and buffalo requires genetic selection and breeding techniques. Understanding the genetic regulation of buffaloes' milk production is essential. By improving breeding methods, this knowledge aids

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in raising productivity and quality features. Although many genes linked to lactation have been discovered, some of their activities are still unknown. In this context, the MAPK15 gene, also known as ERK7, is a serine/threonine kinase within the MAP kinase family, it is currently a focal point of interest owing to its pivotal involvement in cellular processes, including growth, differentiation, transcriptional regulation, and stress response [3]. Despite ongoing elucidation of the precise role of the MAPK15 gene in these processes, recent research suggests that MAPK15 may significantly impact milk production and composition traits [4]. The MAPK-signalling pathway is linked to cell proliferation following growth factor stimulation within the mouse mammary gland [5]. During lactation, the MAPK15 gene functions by inhibiting the activation of glucocorticoid receptors. Given the essential role of glucocorticoids in maintaining lactation, this mechanism underscores how MAPK15 influences the regulation of milk production. The MAPK15 gene works during lactation by preventing glucocorticoid receptor activation. This approach highlights how MAPK15 affects the control of milk supply, which is crucial given the significance of glucocorticoids in sustaining lactation [6].

As far as we know, previous research has yet to explore the variations of the MAPK15 gene and their relationship with milk production and quality characteristics in Murrah buffaloes. [4]. Considering this void, the current study sought to evaluate these genetic differences and investigate possible connections between particular genotypes and milk production and composition characteristics within the MAPK15 gene in Murrah buffaloes. The present study investigated MAPK15 gene polymorphism, including its exons, their flanking intronic regions for milk yield, and milk composition traits through PCR-SSCP and DNA sequencing in *Bubalus bubalis*. The gathered sequence data was utilized to explore various innovative, potential causative MAPK15 variations linked to milk fat percentage and yield.

MATERIALS AND METHODS

All animal-based research was conducted by the Institute's Animal Ethics Committee's criteria of ICAR-NDRI, Karnal, India.

Experimental animals and DNA samples

The genetic diversity present in *Bubalus bubalis* MAPK15 was examined across a sample of 100 Murrah buffaloes. Information collected from the Murrah buffalo population, which boasts a documented lineage and is housed at the Buffalo Research Station in Venkataramannagudem, Sri Venkateswara Veterinary University (SVVU), Tirupati, Andhra Pradesh. The documentation encompassed details regarding the sire, dam, calving season and year, 305-day milk output, cumulative milk output, 305-day fat output, and overall fat output. The buffaloes ranged in age from three to nine years (*i.e.*, 2nd to 8th parity) and grazed on a mixture of ryegrass and white clover pastures throughout their lactation period. All buffaloes were kept under conventional management practices, complying with the Minimum Standard Protocols set forth by the Department of Dairying, Animal Husbandry and Fisheries, Government of India.

Milk sampling and phenotype measurement

All Murrah buffalo females were milked twice daily, and the daily milk production in kilograms of fresh weight was noted using the Lacto Star device (Funke-Gerber, Germany). Approximately 30 ml of milk samples were gathered to assess the fat content employing Gerber's technique [7].

Blood collection and isolation of DNA

Approximately 10 ml of blood was gathered sterilely into a 0.5% EDTA vacutainer tube. The tubes were kept at 4°C until the DNA was extracted. Genomic DNA was retrieved from the blood within 24 hours of collection using the high salt technique [8]. The DNA's concentration and integrity were evaluated using a Nano drop spectrophotometer (Bio-Rad Smart Spec Plus, California, USA). Samples with an optical density (O.D.) ratio of 260/280 ranging from 1.7 to 1.8 were selected and subjected to further analysis through 0.8% and 1% agarose gel electrophoresis, followed by visualization with a gel documentation system. The DNA working solution was created by diluting the stock (100 µL) to a 100 ng/µL concentration for application in polymerase chain reaction (PCR).

Primer design and polymerase chain reaction amplification of MAPK15 gene

To identify polymorphisms in the MAPK15 gene, eight pairs of forward and reverse oligonucleotide primers were crafted (Table 1) based on the *Bos taurus* MAPK15 gene sequence NC_037341.1 obtained from the GenBank repository, utilizing Primer-3 (V.0.4.0) software. PCR amplification (25 µL, final volume) was conducted in a thermal cycler (BioRad, Hercules, CA, USA) utilizing 50 ng of bovine genomic

DNA, 1X PCR buffer, 1.8 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pM of every primer, and 1 U of Taq DNA polymerase. The annealing temperature (T.A.) was fine-tuned during the PCR amplification procedure for each primer sequence (Table 1). The thermocycler (Bio-Rad, USA) protocol consisted of an initial denaturation at 94°C for 2 minutes, succeeded by 35 cycles of denaturation at 94°C for 30 seconds with varying annealing temperatures specific to the primer set (Table 1), extension at 72°C for 1 minute, followed by a concluding extension at 72°C for 10 minutes. The amplified PCR products were placed into the wells of 1.5% and 2% agarose gel with a standard 100 bp DNA ladder (GenerRuler, MBI Fermentas, St. Leon-Rot, Germany) serving as a marker to verify the size of the fragment. Electrophoresis was conducted at 6 V/cm in 1X TBE buffer. Gels were stained with ethidium bromide, visualized under ultraviolet light, and documented using a gel documentation system (Fig.1) (Bio-Rad, Hercules, CA, USA).

Single-strand conformation analysis and sequencing

Genotypic sample examination was executed using single-strand conformation polymorphism (SSCP) techniques. Amplified PCR outputs (10 µL) were segregated via electrophoresis on a 10% native PAGE gel [10% acrylamide: bis-acrylamide (29:1, Sigma Aldrich, USA)] using a 10 µL denaturing agent (95% formamide, 10 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue, 20 mM EDTA Sigma Aldrich, USA) and underwent heat denaturation at 95°C for ten minutes. After denaturation, the PCR outputs were placed on an ice pack and stored at -20°C for 10 minutes. Vertical gel electrophoresis (Bio-Rad, USA) was performed in a Cell electrophoresis unit employing 1X TBE buffer at 200 V for 12 hours at 4°C. For the assessment of SSCP patterns, the gels were silver-stained [9, 10] and captured with a digital camera. The gels were visualized (Fig. 1) and documented using a gel documentation system (Gel doc 1000, Bio-Rad, USA). Band patterns were analyzed based on the number of bands and shifts in mobility and were scored manually (Fig. 2).

Nucleotide sequencing of SSCP alleles

To validate the mobility transition within each configuration, PCR results of every SSCP arrangement in duplicates were selected and sequenced explicitly in both orientations utilizing an automated DNA sequencer (Eurofins Pvt. Ltd., Bengaluru) to pinpoint the genetic variations. The MAPK15 reference sequences from 6 mammalian species (*Bos indicus, Bos taurus, Capra hircus, Bubalus bubalis, Ovis aries, Eqqus caballus,* and *Camelus bactrians*) were retrieved from the GenBank database for comparative analysis. Sequences were analyzed with DNA Baser and Clustal W software [11] to detect SNPs in the MAPK15 gene, aligning them against the Taurine ENSEMBL reference sequence due to the unavailability of the *Bubalus bubalis* base sequence. The MAPK15 sequences across various species were examined utilizing the 'MegAlign (MEGA7)' tool from Lasergene Software (DNASTAR, Madison, WI, USA) to construct a phylogenetic tree (Fig. 3) [9].

Statistical analysis

Data regarding the performance of 100 Murrah buffaloes was gathered and categorized based on herds, years, and seasons. The analysis encompassed the effects of several non-genetic factors such as season (November-February; winter, March-June; summer, July-October; rainy), parity, and the impact of diverse management strategies in both farm and field settings. Statistical evaluations were performed using SPSS 22 software (IBM SPSS Inc., Chicago, IL, USA). To confirm normal distribution, outliers were eliminated through standard plots (Q-Q plot and bar plot), considering only data within the mean ± 2 SD range. For genetic research, 17 sires with two or more progeny were included. The impact of non-genetic factors was adjusted through least squares analysis (LSA) executed in SAS software (Version 9.2). The association analysis of genotypes with milk production attributes was statistically assessed, accounting for farm, season, and parity influences utilizing the fixed General Linear Model (GLM) of SPSS V.22 (SPSS Inc., Chicago, IL, USA). Fixed model used:

$$Yijlmn = \mu + S_i + POC_j + SOC_l + AFC_m + G_n + e_{ijlmn}$$

Y_{ijlmn} represents the recorded quantity of milk produced during the j^{th} calving period, within the l^{th} season of calving, and the mth category of age at first calving (AFC). The term Gn indicates the constant effect associated with the nth SSCP pattern derived from the fourteen exons, µ symbolizing the average mean. Si denotes the i^{th} sire (where i ranges from 1 to 17), POC corresponds to the jth calving period (with l taking values from 1 to 4), while SOC refers to the l^{th} calving season (November to February as winter = 1,

March to June as summer = 2, and July to October as rainy = 3). Additionally, AFC pertains to the mth age at first calving (n varying from 1 to 5), and e_{ijklmn} represents the randomly occurring residual error linked to each observation, which follows a normal distribution with an average of zero and a variance of one.

RESULTS AND DISCUSSION

A PCR product for the MAPK15 gene, measuring 1892bp as anticipated, was successfully amplified, covering all fourteen exons along with the adjacent intron and untranslated regions in 100 Murrah buffaloes. The polymorphism of the MAPK15 gene was investigated using eight pairs of forward and reverse primers via PCR-SSCP analysis. PCR-SSCP examination of amplicons from each exon displayed a uniform pattern, suggesting that all exons were monomorphic (Fig. 2). The purified PCR products were subsequently submitted in duplicates for custom sequencing (Eurofins Pvt. Ltd., Bengaluru) and analyzed. Additionally, to identify nucleotide variations in the MAPK15 gene, reference sequences of *Bos taurus* were aligned and compared with the edited sequences of Murrah buffaloes using Clustal W software. The present investigation revealed no nucleotide variations in the exons of the MAPK15 gene. Nucleotide sequence alignment of this MAPK15 locus showed 100% identity with Indian cattle and water buffaloes and 98% with *Bos taurus* (Fig. 3, Table 3). In this investigation, the calculated least

Table 1. Details of primer sequences (5'to 3' sequences) used for amplification. [Primers sequence, annealing temperature and size of the amplified fragments of Mitogen-Activated Protein Kinase 15 (MAPK15) gene in Murrah buffaloes, base pairs (bp)].

Exon	Primer sequence (5' to 3')	Amplicon length (bp)	Annealing temperature (^oC)	SSCP condition
Exon 1	F- AAGGCAACCGGGTTCAACAG R- AAGTTCCCTGTGAGTAGGGC	292bp	50.0	18^0C, 80V,10h
Exon 2, 3, 4	F- CCTATGGCATCGTGTGGAAG R- GGTAAATGTCCCTGTCGTTCTC	560bp	54	18^0C, 80V,8h
Exon 5, 6	F- ACCCACAAACGCTACATCTTCT R- ACTACGAGTGGGTTCTGGGACT	485bp	54.8	18^0C, 100V,10h
Exon 7	F- GGTGGACATGTGGAGTCTGG R- CTCTGGACCCTGCTCATCC	254bp	56	18^0C, 120V,8h
Exons 8	F- CTCCTGAGTACACTGCCTTCTG R- ACCAACAGTTACCCAGTTCAGG	211bp	56	18^0C, 80V,8h
Exon 9,10,11	F-CAGACGCTAGATGCCCTCCT R- ACACATCGGCCACCAGAC	626bp	54.3	18^0C, 120V,10h
Exon 12, 13	F- CCTCCCAGGCAGAACTCA R- CAGCGGAAGGGAAGGAAG	370bp	52.6	18^0C, 100V,8h
Exon 14	F- GTCTGTGTGCAGGTTCCTCCT R- TATTGGAGGAAGTGATGGAGGT	422 bp	54.7	18^0C, 80V,8h

Table 2. Assessment of evolutionary divergence in the exon sequences of MAPK15 gene between *Bubalus bubalis* and other ruminant species.

Species	*Bos_ taurus*	*Bos_ indicus*	*Capra_ hircus*	*Bubalus_ bubalis*	*Ovis_ aries*	*Equus_ caballus*	*Camelus _bactrianus*
Bos_taurus	1						
Bos_indicus	0.0000	1					
Capra_hircus	0.0047	0.0047	1				
Bubalus_bubalis	0.0047	0.0047	0.0095	1			
Ovis_aries	0.0070	0.0070	0.0023	0.0070	1		
Equus_caballus	0.0298	0.0298	0.0358	0.0298	0.0389	1	
Camelus_bactrianus	0.0329	0.0329	0.0390	0.0329	0.0422	0.0262	1

Table 3. Per cent sequence identity of MAPK 15 gene of *Bubalus bubalis* with different ruminant species.

SI. No.	Species	Sequence description	Accession no.	Per cent Similarity	Reference
1.	*Bos taurus*	Mitogen-activated protein kinase 15 (MAPK15), mRNA	NM_001046110.1	100	[26]
2.	*Bos indicus*	Mitogen-activated protein kinase 15 (MAPK15), mRNA	XM_019973698.1	100	[27]
3.	*Capra hircus*	Mitogen-activated protein kinase 15 (MAPK15), transcript variant X1, mRNA	XM_018058691.1	99	[28]
4.	*Bubalus bubalis*	Mitogen-activated protein kinase 15 (MAPK15), mRNA	XM_006045175.1	99	[29]
5.	*Ovis aries*	Mitogen-activated protein kinase 15 (MAPK15), transcript variant X1, mRNA	XM_004011907.2	98	[30]
6.	*Equus caballus*	Mitogen-activated protein kinase 15 (MAPK15), mRNA	XM_014728166.1	92	[31]
7.	*Camelus bactrianus*	Mitogen-activated protein kinase 15 (MAPK15), transcript variant X1, mRNA	XM_010961158.1	91	[32]

square means for the average milk yield (Kg) and fat percentage (%) over a 305-day lactation period were discovered to be 1189.73 ± 22.03 and 7.88 ± 0.03, correspondingly.

An array of methods exists for identifying novel mutations and polymorphisms. These encompass PCR Restriction Fragment Length Polymorphism (PCR-RFLP), Denaturing Gradient Gel Electrophoresis (DGGE), Protein Truncation Test (PTT), Allele-Specific Oligonucleotides (ASO), and DNA microarray technology [12]. Notably, nucleotide sequencing is a valuable alternative for directly examining sequence variation. Moreover, Single-Strand Conformational Polymorphism (SSCP) is a frequently utilized approach for screening Single-Nucleotide Polymorphisms (SNPs) [12]. A SNP represents a change in a DNA sequence occurring when a solitary nucleotide - A, T, C, or G - varies among species members or within homologous chromosomes of a single organism. They act as biological indicators that are essential in studies examining genome-wide associations. SSCP is highly sensitive in detecting single base pair changes (SNPs) in DNA sequences. It can identify subtle differences in sequence that can affect gene function or predisposition to diseases. SSCP is valued for its sensitivity, cost-effectiveness, and versatility in identifying SNPs and other genetic variations. It continues to be a valuable tool in research and clinical applications related to genetic screening and diagnostics. The SSCP method initiates with PCR amplification of the target gene, subsequently analyzing PCR products to uncover DNA variations employing confirmation-driven mutation scanning techniques. Confirmed PCR products are then sequenced to pinpoint Single-Nucleotide Polymorphisms (SNPs) accurately.

Many factors, including management practices, food, health, and genetic and epigenetic factors, influence the very dynamic physiological process of lactation. Advancing genetic features related to milk production requires understanding the complex biochemical mechanisms underlying the entire lactation cycle. During the lactation curve, this includes the beginning

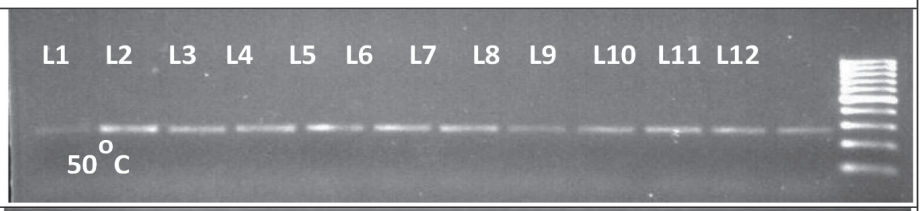
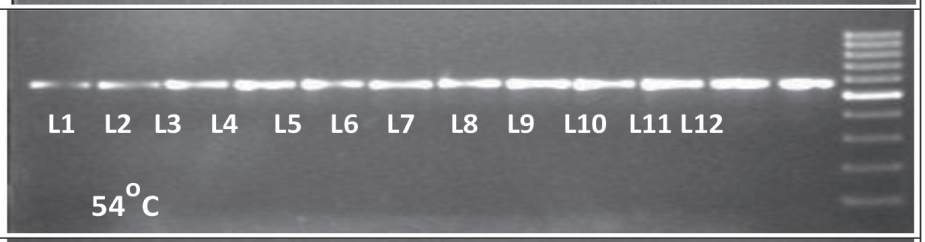
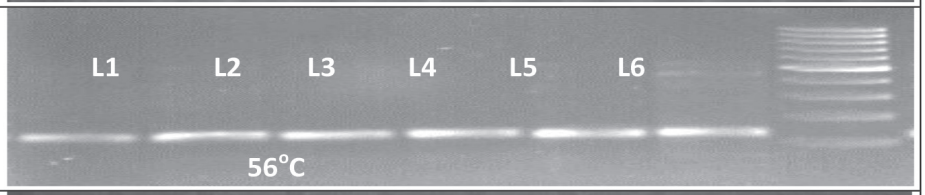
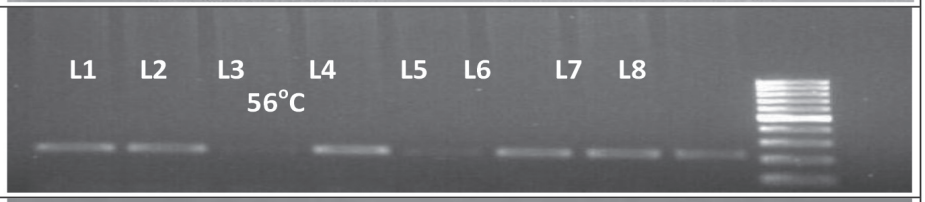
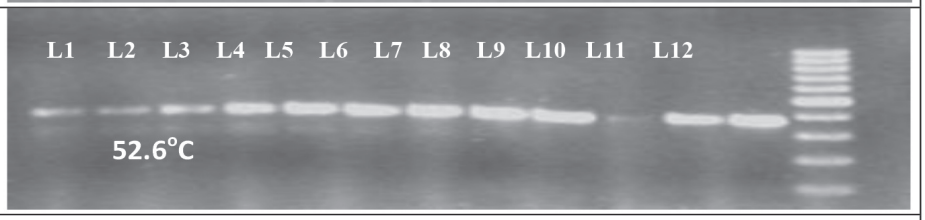
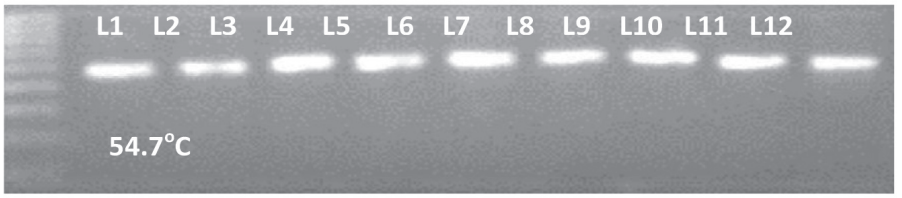
Amplicon with product size	Annealing temp (°C)	Resolution of PCR amplified product on 1.5 % and 2% agarose gel with the selected annealing temperature for each set
Exon 1 (292bp)	50	
Exon 2, Exon3 & Exon4 (560bp)	54	
Exon 5& Exon 6 (485bp)	54.8	
Exon 7 (254bp)	56	
Exon 8 (211bp)	56	
Exon 9, Exon 10 & Exon11 (626bp)	54.3	
Exon12 & Exon 13 (370bp)	52.6	
Exon 14 (422bp)	54.7	

Fig. 1. Details of product size, annealing temperature and gel photographs showing amplification of gene fragments at different annealing temperatures of MAPK 15 gene.

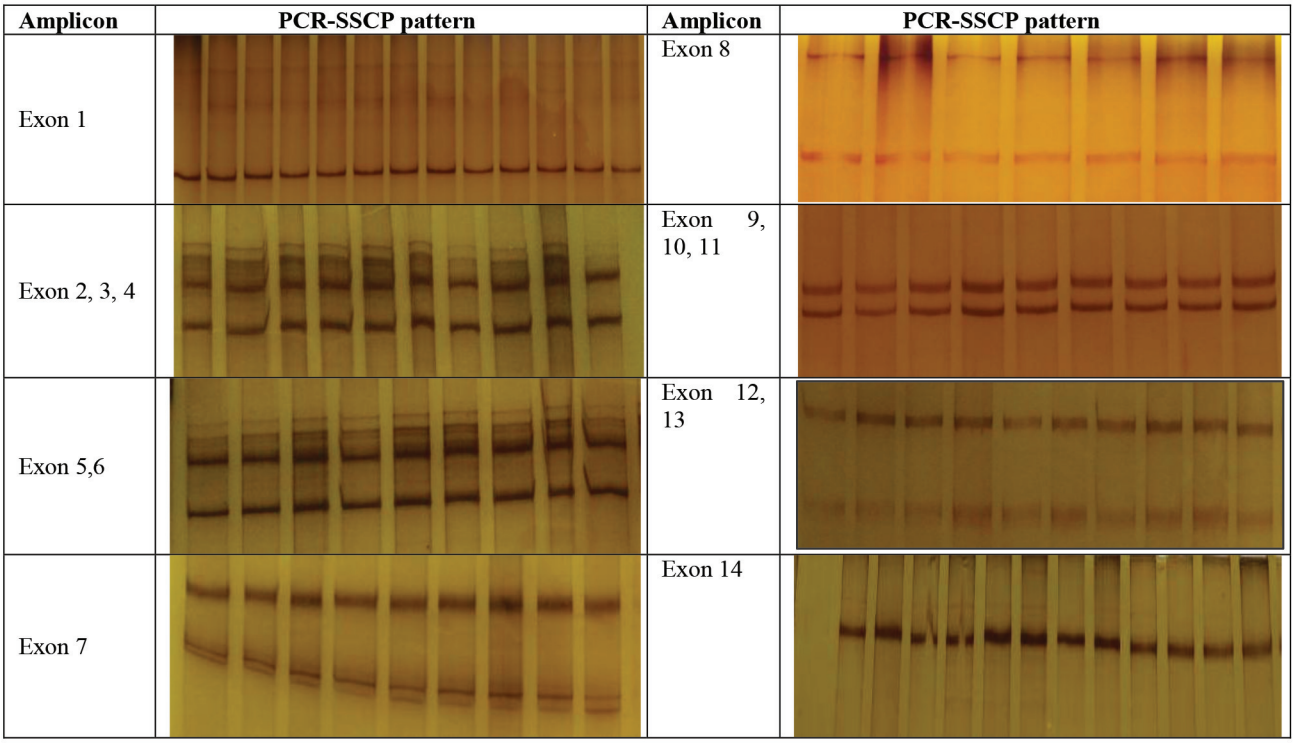


Fig. 2. PCR- SSCP pattern of MAPK 15 gene in Murrah buffaloes.

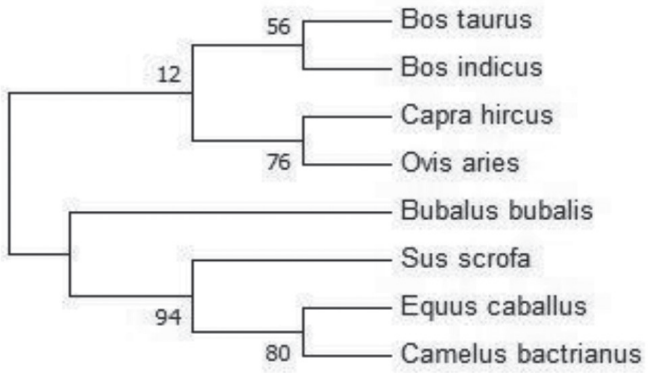


Fig. 3. The NJ tree constructed from pair wise sequence alignment of MAPK15 gene of *Bos taurus* with other species. (The numbers on the node were the bootstrap percentage value from 1,000 times).

and peak stages and the decrease and termination of milk production. Many genes are involved in the lactation process, which controls milk output and composition characteristics.

The genetic assessment of milk production performance in lactating animals relies on molecular technology for identifying genes associated with milk yield and composition efficiency and analyzing the polymorphism of these marker genes. Given the variety of roles and effects that ERKs play in controlling milk's composition and production processes, it is reasonable to assume that genes like MAPK15, which are involved in their synthesis and function, can be considered as possible indicators of milk yield and composition characteristics. According to the literature, the MAP kinase pathway is critical in signalling within mammalian cells during lactation, releasing numerous cytokines upon activation during parturition. MAPK15, a proline-directed serine/threonine protein kinase, undergoes activation in response to diverse extracellular stimuli, including amino acids. The MAPK pathway has the potential to influence the functionality of STAT5a. Evidence suggests that MAPK1, a member of the MAP kinase family similar to MAPK15, enhances protein synthesis [13]. Although many studies have investigated the relationship between MAPK15 and milk production and milk composition in cows, more research should be done on the MAPK15 gene in buffaloes [14, 15, 16]. Consequently, this research sought to elucidate the variations of the MAPK15 gene and investigate its correlation with milk yield and quality characteristics in Murrah buffaloes.

By altering the development of bovine mammary glands, Jiang *et al.* [17] provided insight into how MAP3K1 may impact milk composition features. Additionally, they discovered that animals with the del/del genotype of the MAP3K1 gene's indel polymorphism produced more milk, fat, and protein.

MAP3K1 is thought to be a metabolic trigger that might encourage cell proliferation and is known to be engaged in the MAPK signalling pathway [18, 19].

Mena *et al.* [20] highlighted that MAPKs are essential in multiple cellular functions, particularly in controlling gene expression in heifers. Previous studies highlight the pivotal role of the MAP kinase pathway in mammalian cell signalling during lactation, triggering the release of multiple cytokines during parturition [21]. MAPK15, characterized as a proline-directed serine/threonine protein kinase, is activated in response to a range of extracellular stimuli, notably amino acids [13]. The MAPK pathway could influence the functionality of STAT5a. Evidence suggests that MAPK1 can enhance protein synthesis [22].

Similarly, [23] and [24] identified a notable link between MAPK8IP1, found on chromosome BTA14, and traits related to milk composition and production in dairy cattle. Moreover, [24] uncovered that MAP3K1, situated at 20 Mb on BTA 20, demonstrated a significant connection with milk production traits in Sahiwal cattle as determined by selection signature analysis. According to earlier research, several interrelated spatiotemporal gene expressions and signalling pathways intimately govern changes in physiological function during milk letdown [25]. However, due to contradictory results or a lack of study, the precise function of the MAPK15 gene in controlling milk production and composition features still needs to be discovered.

In contrast to previous studies, our investigation did not identify any SNPs within the MAPK15 gene in Murrah buffaloes, possibly due to the genetic homogeneity of the studied population. The variability in results may stem from species and breed distinctions, population and sample sizes, environmental factors, mating strategies, geographic effects, and genetic variant frequency distributions. Present findings indicate that the sequence of MAPK15 is highly conserved in Murrah buffaloes.

CONCLUSION

MAPK15, which codes for Mitogen-Activated Protein Kinase (MAPK), the enzymatic element of the ERK complex, surfaces as an intriguing candidate gene for lactation traits in dairy livestock. Finding MAPK15 genetic variations in dairy cows has not received enough attention, even though MAPK15 variation significantly influences milk production and composition traits. Present findings of the PCR-SSCP investigation showed a monomorphic pattern, which suggests that polymorphism is unlikely to exist and that Murrah buffaloes are well conserved. The MAPK15 gene's constant monomorphic pattern demonstrated in this study could serve as a unique marker for differentiating the Murrah breed from other buffalo breeds. The present herd under research may have little genetic variety, which might explain the observed monomorphism. Therefore, comprehensive research involving significantly larger and more diverse herds of dairy buffalo breeds is crucial. Further investigation and validation in a larger cohort are necessary to elucidate the exact mechanism and enable the selection of buffalo cows based on desired milk composition traits.

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