Genetic variability in Serum Amyloid A2 (*SAA2*) gene in Murrah buffaloes

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Journal of Livestock Science (ISSN online 2277-6214) 16: 599-605
Received on 28/2/25; Accepted on 3/10/25; Published on 8/10/25
doi. 10.33259/JLivestSci.2025.599-605

Abstract

The present study was carried out to explore the genetic variability in the serum amyloid A2 (*SAA2*) gene in Murrah buffaloes. A total of 100 blood samples were collected from the Murrah buffaloes maintained at Department of Livestock Production and Management, LUVAS, Hisar. The focus of the study was on the intron 1 region of the *SAA2* gene, which spans 516 base pairs. Polymerase chain reaction (PCR) technique was employed to amplify this specific region and restriction fragment length polymorphism (RFLP) technique was utilized to screen for genetic variations. This involved digesting the amplified fragments *with* two restriction enzymes, viz., *HinfI* and *AvaII*. However, RFLP analysis revealed monomorphic patterns for the targeted region of *SAA2* gene using both enzymes, indicating lack of genetic variability at this locus. To further investigate genetic diversity in the targeted region of *SAA2* gene in Murrah buffaloes, 50 samples were Sanger sequenced. Multiple alignment of sequenced samples was performed using BioEdit software. Sequencing results were compared with the reference sequence of *Bos taurus* (NC_037356.1). This comparison revealed nucleotide changes at 15 different positions in the Murrah buffaloes. Among these changes, 9 were transition changes, while 6 were transversion changes. Additionally, insertion of the nucleotide "**A**" was observed at g.26458189 position, and deletion of nucleotide "**G**" was observed at g.26458412 position. These findings suggest that *SAA2* gene in Murrah buffaloes may lack genetic diversity in the region screened by PCR-RFLP but the identified nucleotide changes observed through sequencing could serve as valuable genetic markers for future selection programs.

Keywords: Buffalo; Murrah; *SAA2*; PCR-RFLP; Polymorphism; Sequencing

Introduction

Livestock rearing is one of the most important economic activities in the rural area of the India contributing significantly to the national economy. Buffaloes in the country are the third major contributor to the livestock population and second-largest contributor in production of milk. Milk production in the country has increased by 3.78% over previous year to reach 239.30 million tonnes and about 45% of total milk production in the country is contributed by Indigenous/non-descript buffaloes (BAHS, 2024). According to the 20th Livestock Census, there are 109.85 million buffaloes in the nation, which represents a 1.0% rise over the previous census and contribute around 20.45% of India's total livestock population. Out of total buffaloes, 20.3% are pure bred, 34.3% are graded and the remaining 45.4% are in the non-descript category (Livestock Census, 2019). There are 21 buffalo breeds registered in India, and Murrah is one of the best breed (NBAGR, 2025). In terms of population Murrah buffalo contribute around 65% of total registered breeds of buffaloes (Chandra Prasad et al., 2019). Total population of Murrah in the country is about 47 million which comprise 42.8% of total buffalo population (Livestock census, 2019). “Murrah” breed is also known as “Delhi”, “Kundi” and “Kali”.

The economic viability of the dairy industry is basically depending on production performance and reproductive efficiency of dairy animals. Marker-assisted selection (MAS) is an indirect selection approach wherein desirable traits are selected based on molecular markers that are tightly linked to genes or quantitative trait loci (QTL) governing those traits. The effectiveness of MAS relies on accurately identifying and validating associations between genetic markers and the corresponding QTL. MAS has become a powerful tool in accelerating genetic improvement, especially for complex traits that are difficult to measure or have low heritability. In livestock selection programs, MAS improves the accuracy of selection of economically important traits. Marker information can be used to predict an animal’s performance before the records of the performance traits are available. Molecular markers for improving milk production in dairy animals have showed to be helpful to animal breeders for planning appropriate breeding programme (Kumar et al*.,* 2020).

The bovine serum amyloid A2 (SAA2) gene is located on chromosome 29 of the bovine genome and is a member of the highly conserved serum amyloid A (SAA) protein family. In mammals, this family comprises five isoforms SAA1, SAA2, SAA3, M-SAA3.2, and SAA4 each exhibiting diverse biological functions (Kovacevic-Filipovic et al., 2012). Yang et al. (2015) reported suggest that SAA2 gene may function as a regulatory adaptor protein involved in lipid synthesis and metabolic processes, as well as in the maintenance of mammary gland tissue, mechanisms that are closely associated with milk fat synthesis and composition. Study shows a statistically significant effect of genetic variability in *SAA2* gene (c.-84 G>C) on milk constituents as well as genetic variability (c.114 G>A) significantly affecting calving interval in dairy cattle (Luczak and Piatkowska, 2021). However, till date, there are no published reports pertaining to study of polymorphism in *SAA2* gene in Murrah buffaloes, therefore, the current study was conducted to identify genetic variations in intron 1 region of *SAA2* gene in Murrah buffaloes.

Materials and methods

Ethical approval

The authors hereby confirm that they have fully complied with the ethical policies and have obtained the necessary approval for collection of blood samples from Institutional Animal Ethics Committee (IAEC) of LUVAS, Hisar, Haryana, India (VCC/IAEC/376-97, 03-02-22).

Study site and sample collection

To analyze the polymorphism in intron 1 region of *SAA2* gene, blood samples were collected from 100 Murrah buffaloes maintained at Buffalo Farm, LUVAS, Hisar and stored carefully in Animal Cytogenetics laboratory. The city is situated in semi-arid region and climatic condition is sub-tropical in nature. Hisar is located at a specific geographical coordinate: 29° 10’ north latitude and 75° 40’ east longitude, with an elevation of 215.2 meters above sea level.

DNA extraction

DNA extraction was performed using the Maxwell RSC genomic DNA extraction kit. This kit utilizes a unique technology involving microscopic magnetic particles that selectively bind to DNA molecules, facilitating efficient purification and isolation of high-quality DNA from the samples.

Quality and quantity check of DNA

The quality of the extracted DNA was evaluated by subjecting it to agarose gel electrophoresis. To achieve this, a 1% agarose suspension was prepared in 1X TAE buffer, which had a pH of 8.0. Ethidium bromide was added to the agarose gel solution at a concentration of 3 µl per 100 ml. Following solidification, the gel was carefully

transferred to an electrophoresis chamber containing 650 ml of 1X TAE buffer. Then, 2 µl of DNA was mixed with 1 µl of 6X gel-loading dye and loaded into separate wells. Electrophoresis was performed at a voltage of 80 V for 45 minutes at room temperature. After completion, the gel was carefully removed from the chamber and placed on a transilluminator. The DNA bands were visualized under UV light, and photographs were captured using a gel documentation system. The presence of sharp bands indicated accurate DNA concentration. Additionally, the quality and quantity of DNA were assessed using a Nanodrop spectrophotometer. The optical density was measured at wavelengths of 260 nm and 280 nm, with distilled water serving as the blank sample. The quantity of DNA was calculated using a specific formula.

Quantity of DNA in µg/ml = OD260 × 50 × Dilution Factor

The ratio between OD260 and OD280 was observed for each sample. DNA samples having a range of 1.7 to 1.9 were considered as good and used for the further study.

Primers used and PCR conditions

In the present study, the targeted region of intron 1 in Murrah buffaloes was amplified using primers previously reported by Luczak and Piatkowska (2021). The forward primer, 5'AAACGCATGGCTGAGAGACT3', and the reverse primer, 5'GACAGGGGTGAGGAGAACAG3', yielded an amplicon size of 516 bp. The polymerase chain reaction (PCR) was performed in a 25 µl reaction volume, comprising 2.0 µl of DNA template, 1.5 µl each of forward and reverse primers, 12.5 µl of green master mix (Promega), and 7.5 µl of nuclease-free water. The amplification cycling conditions for intron 1 involved an initial denaturation step at 95°C for 3 minutes, followed by 34 cycles of denaturation at 95°C for 45 seconds, annealing at 57.5°C for 30 seconds, and extension at 72°C for 1 minute and 30 seconds. A final extension step was performed at 72°C for 10 minutes

RFLP conditions

For the Restriction Fragment Length Polymorphism (RFLP) analysis, 10 µl of each PCR-amplified product was subjected to enzymatic digestion with 5 units of the *HinfI* restriction enzyme, which recognizes the 5' G/ANTC 3' sequence, and 5 units of the *AvaII* restriction enzyme, which recognizes the 5' G/GWCC 3' sequence. The digestion reaction was performed in a total volume of 30 µl and incubated in a water bath at a temperature of 37°C for a period of 16 hours. Following digestion, the resulting fragments were separated by electrophoresis on a 2% agarose gel prepared in 1% TAE buffer. The separated fragments were then visualized using a gel documentation system, allowing for the detection of any polymorphisms present in the digested DNA fragments.

Sanger sequencing

To further investigate the genetic variations, present in the intron 1 region, 50 PCR-amplified samples were subjected to Sanger sequencing. The resulting sequences were then analyzed using multiple sequence alignment techniques, employing both BioEdit and ClustalW software. This alignment process involved comparing the obtained sequences to the reference sequence of *Bos taurus* (NC_037356.1), allowing for the identification of any nucleotide variations or polymorphisms present in the intron 1 region of the Murrah buffalo samples.

Results

A 516 bp fragment of the intron 1 region of the *SAA2* gene was successfully amplified using PCR, with primer pairs previously reported in the literature. To optimize the PCR conditions, a gradient PCR was performed to standardize the annealing temperature. The optimal annealing temperature for the primer pair specific to the intron 1 region of the *SAA2* gene was determined to be 57.5°C for 30 seconds. The resulting 516 bp PCR product (Plate 1) was then subjected to restriction digestion using the *HinfI* and *AvaII* enzymes individually, at 37°C for 12-16 hours, to detect potential polymorphisms. It was expected that the *SAA2* gene would contain a HinfI restriction site within the 516 bp fragment, resulting in the production of smaller fragments of 288, 176, 112, 80, and 32 bp. However, upon screening the *SAA2*/*HinfI* products from 100 Murrah buffalo samples, it was observed that all samples exhibited an identical restriction pattern, characterized by the absence of the restriction site, resulting in a single 516 bp fragment (Plate 2). This finding indicated a monomorphic pattern, suggesting the absence of genetic variation at this locus. Furthermore, the PCR product was also digested with the *AvaII* restriction enzyme and it was anticipated that restriction digestion will result in production of fragments of 473, 432, 43 and 41 bp. But upon screening *SAA2*/*AvaII* products a monomorphic pattern was revealed, indicating the absence of genetic variation at this locus (Plate 3).

Sequencing of intron 1 region of *SAA2* gene

A total of 50 samples of the Intron 1 region of the *SAA2* gene were outsourced to Biokart India Pvt. Ltd. for Sanger sequencing. The obtained sequences were then aligned using BioEdit software to facilitate multiple sequence comparisons. Upon comparing the sequencing results with the reference sequence of *Bos taurus* (NC_037356.1), a total of 15 nucleotide changes were identified. Among these changes, 9 were classified as transition mutations, while 6 were categorized as transversion mutations. Notably, an insertion of the nucleotide "A" was observed at the

g.26458189 position, and a deletion of the nucleotide "G" was detected at the g.26458412 position. Furthermore, five novel single nucleotide polymorphisms (SNPs) were discovered, specifically g.26458214 A>G, g.26458305 T>A, g.26458385 C>T, g.26458443 G>A, and g.26458444 A>C (Figure 1, 2 and 3).

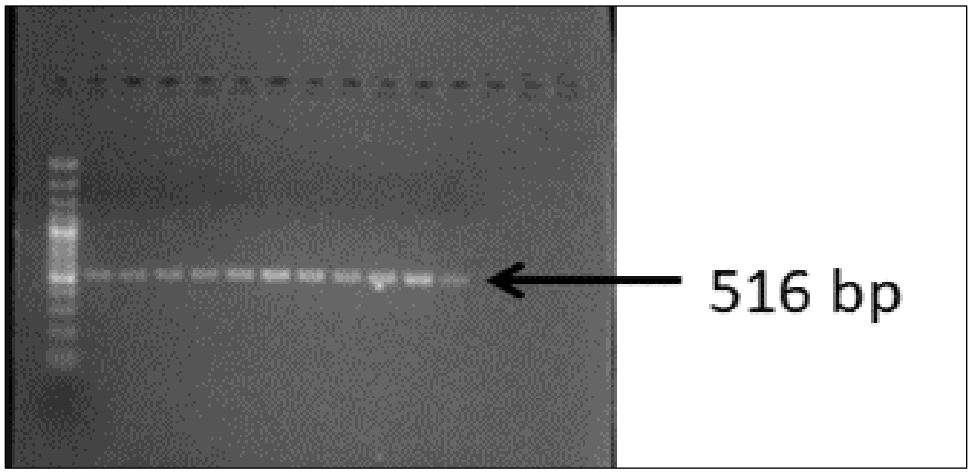

Plate 1: PCR amplified product of Intron 1 region of *SAA2* gene in Murrahbuffaloes (Ladder: 100 bp)

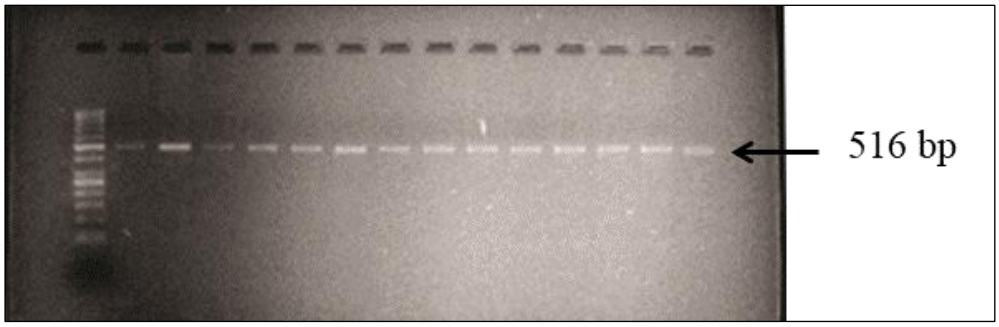

Plate 2: PCR-RFLP product of Intron 1 region of *SAA2* gene (*Hinf*I) in Murrah buffaloes (Ladder: 50 bp)

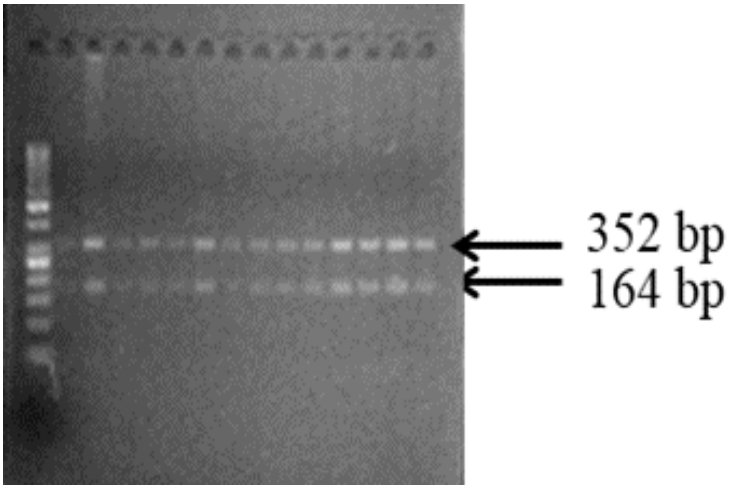

Plate 3: PCR-RFLP product of Intron 1 region of *SAA2* gene (*Ava*II) in Murrah buffaloes (Ladder: 50 bp)

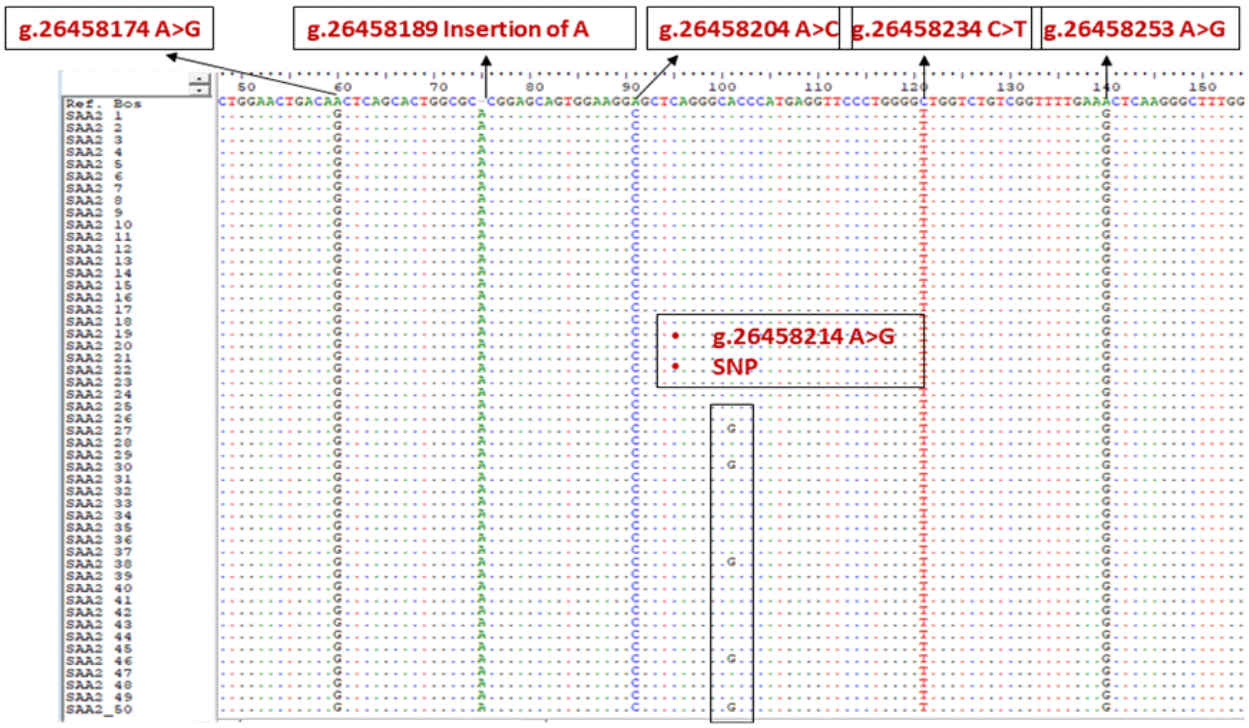

Figure 1: Clustal W analysis of amplified *SAA2* gene sequence in Murrah buffaloes compared with reference sequence of *Bos taurus*

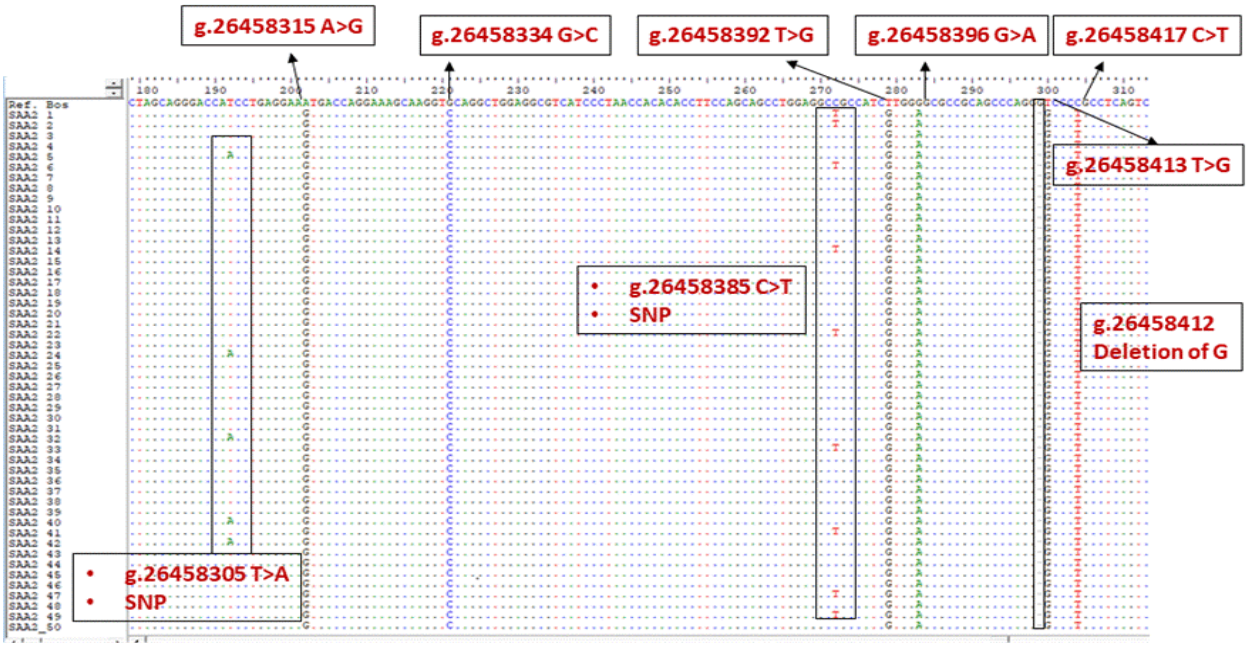

Figure 2: Clustal W analysis of amplified *SAA2* gene sequence in Murrahbuffaloes compared with reference sequence of *Bos taurus*

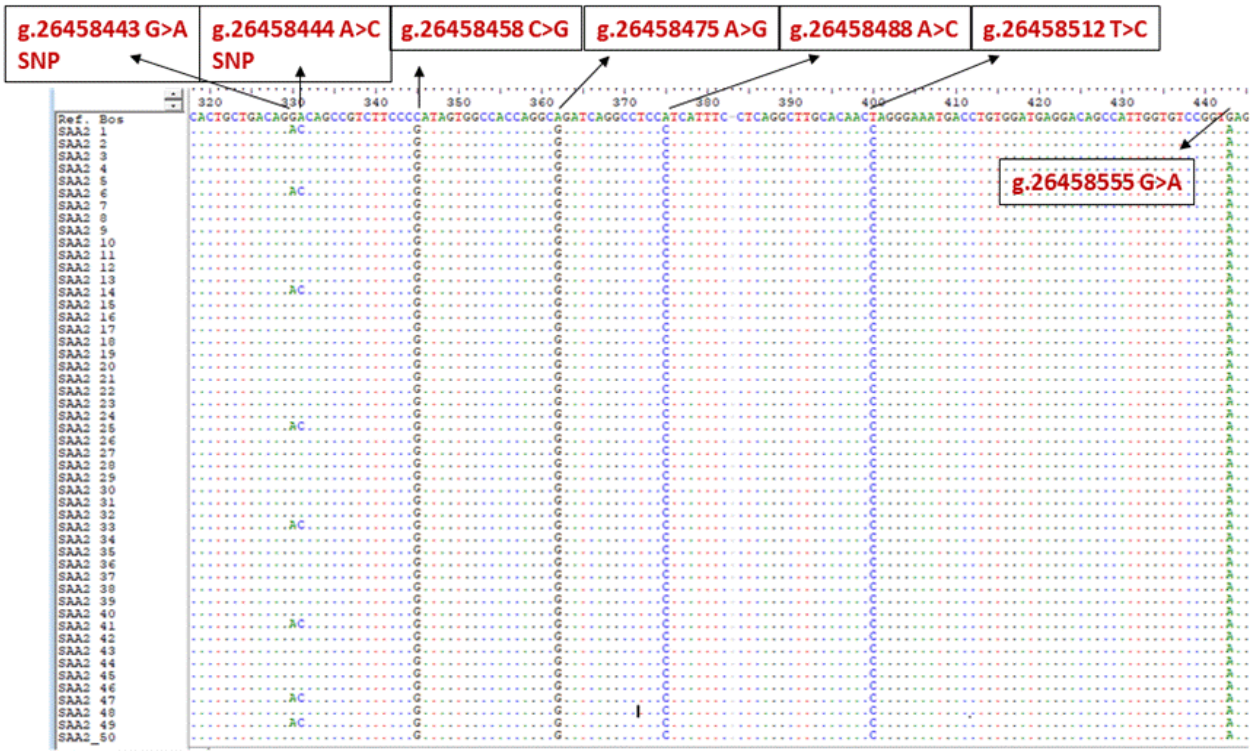

Figure 3: Clustal W analysis of amplified *SAA2* gene sequence in Murrahbuffaloes compared with reference sequence of *Bos taurus*

Discussion

A 516 bp fragment of the intron 1 region of the *SAA2* gene was successfully amplified using PCR with the help of previously reported primers. To optimize the PCR conditions, a gradient PCR was performed to standardize the annealing temperature. Consequently, the optimal annealing temperature for the primer pair specific to the intron 1 region of the *SAA2* gene was determined to be 57.5°C for 30 seconds. Interestingly, this temperature-time combination of 57.5°C for 30 seconds for annealing of the primer was also reported by Raza et al. (2022) in Chinese Holstein cows, indicating consistency in the optimal annealing conditions. However, a slightly different annealing temperature of 60°C was reported by Łuczak et al. (2021), highlighting the possibility of variations in optimal PCR conditions depending on the specific experimental setup or primer design.

The PCR-amplified product of the 516 bp size of the Intron 1 region of the *SAA2* gene was subjected to restriction digestion with the *HinfI* and *AvaII* enzymes individually at 37°C for 12-16 hours to identify potential polymorphisms. Initially, it was expected that the *SAA2* gene would possess a *HinfI* restriction site within the 516 bp fragment, resulting in the production of fragments of 288, 176, 112, 80, and 32 bp. However, upon screening the *SAA2*/*HinfI* products from 100 Murrah buffalo samples, all samples exhibited an identical restriction pattern, characterized by the absence of the restriction site, yielding a single 516 bp fragment. This finding indicated a monomorphic pattern, suggesting the absence of genetic variation at this locus. Furthermore, the PCR-amplified product was digested with the *AvaII* restriction enzyme and it was expected that the gene would possess a *AvaII* restriction site and would produce fragments of 473, 432, 43 and 41 bp but upon screening *SAA2*/*AvaII* products monomorphic patterns were revealed, indicating the absence of genetic variation at this locus. On contrast, Luczak and Piatkowska (2021) reported the presence of polymorphic loci within the intron 1 region of the *SAA2* gene in Polish Holstein Friesian cows, following digestion with the *HinfI* enzyme. They identified a c.84G>C polymorphism, which resulted in the production of fragments of 228, 176, and 112 bp for the 'G' allele and 228, 176, 80, and 32 bp for the 'C' allele. Additionally, they found a c.114G>A polymorphism upon digestion with the *AvaII* enzyme, yielding fragments of 432, 43, and 41 bp for the 'G' allele and 473 and 43 bp for the 'A' allele. Notably, they observed that cows with the c.84G>C polymorphism had a shorter calving interval and lower age at first calving, while cows with the c.114G>A polymorphism produced lower daily milk yields and had the lowest somatic cell count.

Similarly, Yang et al. (2015) reported polymorphisms c.84G>C and c.114G>A in Chinese Holstein cows and found significant associations with estimated breeding values (EBVs) for traits such as milk yield and protein yield. They suggested that the c.114G>A polymorphism, located in the promoter region of the gene, may influence the gene's transcriptional activity. Raza et al. (2022) also investigated the intron 1 region of the *SAA2* gene in Chinese Holstein cattle and identified three single nucleotide polymorphisms (SNPs), namely g.14061A>G, g.14072G>C, and g.14819C>T. They found significant effects of the g.14061A>G and g.14819C>T SNPs on milk production traits.

Conclusion

In summary, the loci examined in Murrah buffaloes using the PCR-RFLP technique in the present study were found to be monomorphic, indicating an absence of genetic variation at these specific loci. However, sequencing analysis of the samples revealed nucleotide changes at distinct locations in Murrah buffaloes compared to Bos Taurus, and these variations can potentially serve as selection signatures in Murrah buffaloes. Notably, the present study provides the first evidence of single nucleotide polymorphisms (SNPs) in the *SAA2* gene of Murrah buffaloes. Although the study identified these genetic variations, association analysis could not be performed due to the limited sample size, highlighting the need for further research with a larger dataset to explore the potential associations between these genetic variations and economically important traits in Murrah buffaloes.

Acknowledgments

Authors are thankful to the funding agency (HSCSIT) for providing financial support for carrying out the research work. The support and facilities provided by Director of Research, LUVAS, Hisar is also fully acknowledged.

Funding statement

Funding for carrying out the research was provided by Haryana State Council for Science, Innovation and Technology (HSCSIT/R&D/2021/2919).

Competing interests

The authors declare that there are no conflicts of interest with this publication.

Ethical standards

The authors confirm the ethical policies of the journal, as noted on the journal's author guidelines page.

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