

# Allelic and genotypic frequencies of polymorphisms associated with productive traits in cattle of the Sardo Negro breed of Mexico

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## Research Article

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# Abstract

Sardo Negro cattle are a Mexican breed recognized for their ability to produce meat and milk in tropical climates. The objective of this study was to estimate the allelic and genotypic frequencies of polymorphisms in the GH, CAST, CAPN1, CSN3, BLG and CSN2 genes by polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) in 80 animals from a herd of Sardo Negro cattle. High frequencies of favorable alleles were detected among the CAST (A: 0.76), BLG (B: 0.86), and CSN2 (A2: 0.99) gene polymorphisms; however, lower frequencies of favorable alleles were detected among the CAPN4751 (C: 0.17), CSN3 (B: 0.07), and GH (V: 0) gene polymorphisms. Genotyping with rapid and low-cost methods, such as PCR–RFLP, could be used to design genetic selection strategies aimed at increasing the frequencies of favorable haplotypes for the production of meat and milk in this population in Mexico.

## Introduction

Sardo Negro cattle are a Zebu breed native to the tropical region of the Gulf of Mexico and are characterized by their predominantly black pigmentation and a high ability to adapt to tropical ecosystems (Martínez-González et al. 2007). Sardo Negro cattle originated through crossings of the Gyr and Indu-Brasil breeds with the purpose of obtaining a highly productive breed for dual-purpose systems under adverse climatic conditions (Marínez-Rocha et al. 2023). Owing to its outstanding capacity for growth and milk production, the Sardo Negro breed is used in production systems in the tropical regions of Mexico (Garduño-López et al., 2024), where it currently has a record of 50,000 animals registered with its pedigree (Domínguez-Viveros et al., 2021). Because the Sardo Negro cattle have been established as a new Mexican breed, understanding the genotypic frequencies and distributions of alleles that were previously associated with productive traits is essential as this population in Mexico comprises a unique reservoir of genes as a result of the selection by local stockbreeders, creating the possibility of using mating strategies to increase the frequencies of favorable alleles in these populations. In cattle, single nucleotide polymorphisms (SNPs) associated with traits of economic interest have been identified, such as those in the growth hormone (GH) gene, which is located on bovine chromosome 19 (Hediger et al. 1990) and plays a fundamental role in the regulation of postnatal growth and metabolism in mammals (Amiri et al. 2018). The V allele of the g.2141C > G SNP in the GH gene is associated with greater daily weight gain in cattle (Hartatik et al., 2020). The calpastatin gene (CAST) is located on chromosome 7 (Bishop et al. 1993) and encodes endogenous inhibitors that are responsible for the regulation of the proteolytic activity of calpains (Calvo et al. 2014). The A allele of the g.2959A > G SNP in the CAST gene is associated with greater meat tenderness (Casas et al. 2006; Morris et al. 2006; Curi et al. 2009). The  $\mu$ -calpain gene (CAPN1) is located on bovine chromosome 29 (Curi et al. 2009) and encodes the synthesis of cysteine protease enzymes that are involved in the postmortem proteolytic degradation of meat (Page et al. 2002). The C allele of the CAPN4751 polymorphism (g.6545C > T SNP) is associated with greater meat tenderness (White et al. 2005; Curi et al. 2009). Another gene that is relevant to milk production is the kappa-casein gene (CSN3), which has been identified on chromosome 6 in cattle (Azevedo et al. 2008) and is essential as a stabilizer of the micellar structure of milk, influencing the processing characteristics of some milk products (Shekar et al. 2006). The B allele of the g.88532332A > C SNP in the CSN3 gene has been associated with an increase in the amount of protein and the percentage of fat in milk, improving the yield of cheese (Tsiaras et al. 2005; Bangar et al. 2021). The gene for beta-lactoglobulin ( $\beta$ -LG) is located on bovine chromosome 11 (Hayes and Petit. 1993) and affects the concentration of caseins in milk (Ganai et al. 2008). The B allele of the g.5305C > T SNP in the  $\beta$ -LG gene is linked to a higher concentration of caseins, which is significantly associated with an increase in the percentage of fat in milk, thus being favorable for the production of cheese (Tsiaras et al. 2005). The beta-casein gene (CSN2) is located on chromosome 6 and is key in the formation of the micellar structure in milk because of its ability to act as a calcium chelator (Li et al. 2022). The A2 allele of the c.245C > A SNP in the CSN2 gene is associated with healthier milk consumption because it prevents the release of  $\beta$ -casomorphin-7,

which is produced during the gastrointestinal digestion of milk with the A1 variant and is associated with milk protein intolerance (Jianlin et al. 2016). Molecular techniques such as polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) can be used to detect the presence of polymorphic variants, which allows the inclusion of favorable alleles in selection programs for this expanding local breed. Therefore, the present study aimed to estimate the allelic and genotypic frequencies of SNPs that are associated with productive traits in Sardo Negro cattle from Mexico.

## Materials and methods

### Animal population and sampling

A total of 80 samples from a herd of Sardo Negro cattle registered by the Mexican Association of Cebu Breeders (Association Mexicana de Criadores de Cebu, AMCC) were used. The herd is located in the municipality of Comala (19°19'32"N, 103°45'18"W) in the state of Colima, Mexico, at an altitude of 706 meters above the sea level (MASL). According to the Koppen classification (Kottek et al. 2006), the climatic condition of the study area is considered a tropical savanna climate (Aw), which is characterized by warm subhumid conditions with summer rain, reaching a maximum temperature of 26.2°C with an average of 24.4°C, while the minimum recorded temperature is 22.9°C, with an average rainfall of 906.9 mm according to the National Institute of Statistics and Geography (INEGI, 2021). Three milliliters of blood was collected from the middle coccygeal vein into Vacutainer® tubes (Becton–Dickinson and Company, USA) containing ethylenediaminetetraacetic acid (EDTA), and the samples were kept refrigerated at 4°C until processing.

### DNA extraction and genotyping

The extraction and purification of genomic DNA were performed with a Qaim® DNA kit (Qiagen, Hilden, Germany) according to the protocol recommended by the manufacturer. The detailed information for each SNP, the sequence of each primer, the optimal annealing temperature, the restriction enzymes used, and the size of each amplicon are described in Table 1. Each sample analyzed consisted of ~ 100 ng of genomic DNA, 2 µL of 1× PCR buffer with 20 mM MgCl<sub>2</sub> (Thermo Fisher Scientific, Inc., MA, USA), 0.5 U of Dreama polymerase (Thermo Fisher Scientific, Inc.), 5 pmol of each primer, and ddH<sub>2</sub>O to obtain a total volume of 15 µL. Amplification was performed on a Techne® TC-5000 thermocycler (Techne Inc., Burlington, NJ, USA) with the following thermal profile: initial denaturation at 94°C for 5 minutes, followed by 30 cycles at 94°C for 20 seconds, an annealing temperature as described in Table 1 for each gene for 20 seconds, an extension at 72°C for 2 minutes, and a final extension of 5 minutes at 72°C. The digested PCR products were separated by electrophoresis on a 4% agarose gel stained with Gel Red™ (Biotium, Hayward, CA, USA). The bands were observed and photographed under ultraviolet light.

### Calculation of frequencies

To estimate frequencies for each allele and genotype, the direct count method was used (Nei, 1987).

Table 1  
Sequences of the oligonucleotide primers and the restriction enzymes used in this study

| Gene<br>(SNP)                                                                                                                                                                             | Sequence (5' → 3')                                                   | T <sub>m</sub><br>(°C) | Restriction<br>enzyme | Fragment<br>length<br>(bp) | Reference                          |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------|------------------------|-----------------------|----------------------------|------------------------------------|
| GH (g.2141C<br>> G)                                                                                                                                                                       | F: CGGACCGTGTCTATGAGAAGCTGAAG<br>R: TTTCTTGAGCAGCGCGTCGTCA           | 58.8                   | <i>AluI</i>           | 428                        | Balogh et al. (2009)               |
| CAST<br>(g.2959A > G)                                                                                                                                                                     | F: TGAATTTGTCTGGTTCACCTGT<br>R: GACAAGGTGCGGAAGTCCTA                 | 63                     | <i>DdeI</i>           | 486                        | Blecha et al. (2019)               |
| CAPN1<br>(g.6545C > T)                                                                                                                                                                    | F: CCTGGAGTCCTGCCGCAGCATGGTCAAC<br>R: AAGCTGCAGGAGCTGCCCCAAGCCAGGC   | 61                     | <i>DdeI</i>           | 334                        | Blecha et al. (2019)               |
| CSN3<br>(g.88532332A<br>> C)                                                                                                                                                              | F: TGTGCTGAGTAGGTATCCTAGTTATGG<br>R: GCGTTGTCTTCTTTGATGTCTCCTTAG     | 59                     | <i>HinfI</i>          | 426                        | Barroso et al. (1997)              |
| β-LG<br>(g.5305C > T)                                                                                                                                                                     | F: TGTGCTGGACACCGACTACAAAAAG<br>R: GCTCCCGGTATATGACCACCCCTCT         | 58                     | <i>HaeIII</i>         | 148                        | Medrano and Aguilar-Córdoba (1990) |
| CSN2 (c.245C<br>> A)                                                                                                                                                                      | F: CCTTCTTTCCAGGATGAACTCCAGG<br>R: GAGTAAGAGGAGGGATGTTTTGTGGGAGGCTCT | 57                     | <i>DdeI</i>           | 121                        | Miluchova et al. (2009)            |
| GH: growth hormone; CSN3: kappa-casein; β-LG: beta-lactoglobulin; CSN2: beta-casein; CAPN1: μ-calpain; CAST: calpastatin; T <sub>m</sub> : annealing temperature; F: forward; R: reverse. |                                                                      |                        |                       |                            |                                    |

## Results

On the basis of the results of the PCR–RFLP assays, it was possible to genotype the bovines included in this study. The resulting polymorphisms of each gene and their restriction fragments of different lengths are listed in Table 2. The images of the agarose gels for the polymorphisms in the GH, CAST, CAPN1, CSN3, β-LG, and CSN2 genes are shown in Figs. 1, 2, 3, 4, 5, and 6, respectively. The genotypic and allelic frequencies obtained are summarized in Table 3.

Table 2  
Lengths of the restriction fragments in each gene by  
genotype

| Gene            | Genotype | Length (bp)               |
|-----------------|----------|---------------------------|
| GH              | LL       | 265, 96, 51, and 16       |
|                 | LV       | 265, 147, 96, 5,1 and 16  |
|                 | VV       | 265, 14,7 and 16          |
| CAST            | AA       | 195, 191, 68, and 32      |
|                 | GA       | 386, 195, 191, 68, and 32 |
|                 | GG       | 386, 68, and 32           |
| CAPN1           | WC       | 213, 63, and 54           |
|                 | TC       | 213, 159, 63, and 54      |
|                 | TT       | 159, 63, and 54           |
| CSN3            | AA       | 326, 100, and 27          |
|                 | BA       | 426, 326, 100, and 27     |
|                 | WB       | 426 and 27                |
| $\beta$ -LG     | AA       | 148 and 99                |
|                 | BA       | 148, 99, and 74           |
|                 | WB       | 99 and 74                 |
| CSN2            | A1A1     | 121                       |
|                 | A1A2     | 121, 86, and 35           |
|                 | A2A2     | 86 and 35                 |
| bp: base pairs. |          |                           |

Table 3  
Estimated allelic and genotypic frequencies in the GH, CAST, CAPN1, CSN3, -LG, and CSN2 genes in Sardo Negro cattle

| $\beta$               |                          |             |             |                    |      |
|-----------------------|--------------------------|-------------|-------------|--------------------|------|
| Gene                  | Genotype frequencies (n) |             |             | Allele frequencies |      |
| GH                    | LL                       | LV          | VV          | F                  | F    |
|                       | 1.00 (80)                | 0.00<br>(0) | 0.00<br>(0) | 1.00               | 0.00 |
| CAST                  | AA                       | GA          | GG          | A                  | F    |
|                       | 0.68 (54)                | 0.18 (14)   | 0.15 (12)   | 0.76               | 0.24 |
| CAPN1                 | TT                       | CT          | WC          | T                  | C    |
|                       | 0.69 (55)                | 0.29 (23)   | 0.03<br>(2) | 0.83               | 0.17 |
| CSN3                  | AA                       | BA          | WB          | A                  | B    |
|                       | 0.85 (68)                | 0.15 (12)   | 0.00<br>(0) | 0.93               | 0.07 |
| $\beta$ -LG           | AA                       | BA          | WB          | A                  | B    |
|                       | 0.03<br>(2)              | 0.24 (19)   | 0.74 (59)   | 0.14               | 0.86 |
| CSN2                  | A1A1                     | A1A2        | A2A2        | A1                 | A2   |
|                       | 0<br>(0)                 | 0.03<br>(2) | 0.98 (78)   | 0.01               | 0.99 |
| n: number of animals. |                          |             |             |                    |      |

## Discussion

### (g.2141C > G SNP)

The studied population of Sardo Negro cattle was monomorphic for the L allele of the g.2141C > G SNP in the GH gene. These results are consistent with those previously described for *Bos taurus indicus* cattle of the Gyr (Pawar et al. 2007) and Nellore (Cardoso et al. 2014) breeds, in which the L allele was predominant. The similarity in the allelic frequencies could be due to the influence of the Zebu cattle genetics, which gave rise to the Sardo Negro breed. The fixation of the L allele in *B. t. indicus* cattle could be due to processes of natural selection (Bangar and Magotra 2021). In crosses of *B. t. taurus*  $\times$  *B. t. indicus* cattle, a frequency of 0.8 was found for the L allele (Hartatik et al. 2020). According to these results, the LL genotype is found less frequently in *B. t. taurus* cattle, whereas the VV genotype is found more frequently and is associated with greater daily weight gain (Bayraktar and Ozmedir, 2022).

### CAST (g.2959A > G SNP)

In the CAST gene, the allelic frequencies of A (0.76) and G (0.24) were determined. In addition, the AA, AG, and GG genotypes had frequencies of 0.68, 0.18, and 0.15, respectively. The frequency of the A allele observed in this study differs from those reported in populations of the Sardo Negro (0.58) and Gyr (0.56) breeds; however, it is similar to those reported in other *B. t. indicus* breeds, such as Brahman (0.72) and Guzerat (0.79) (Parra-Bracamonte et al. 2015). The discrepancy in the frequencies could be due to small sample sizes. Considering that the A allele is associated with greater meat tenderness because of lower calpastatin activity (Salcedo-Uriarte et al. 2021), its high frequency detected in this study could be used as a basis for the development of mating strategies to increase the frequency of this allele in Sardo Negro cattle from Mexico (Parra-Bracamonte et al. 2015).

## **CAPN1 (g.6545C > T SNP)**

For the g.6545C > T SNP in the CAPN1 gene, the T and C alleles were identified, with frequencies of 0.83 and 0.17, respectively. In addition, the TT, TC, and CC genotypes were observed, with frequencies of 0.69, 0.29, and 0.03, respectively. Similar to our study, the C allele was reported to have a low frequency in *B. t. indicus* cattle of the Sardo Negro (0.08), Gyr (0.09), Indu-Brasil (0.04), Brahman (0.08), and Guzerat (0.02) breeds (Parra-Bracamonte et al. 2015); thus, the low frequency of the C allele reported in this study could be because this variant is rare in *B. t. indicus* cattle. According to Salcedo-Uriarte et al. (2021), compared with the T allele, the C allele is associated with greater meat tenderness. Through genotyping of the animals, it could be possible to propose selection schemes and increase the frequency of the C allele, which is associated with meat tenderness, through selective crosses.

## **CSN3 (g.88532332A > C SNP)**

For the g.88532332A > C SNP in the CSN3 gene, the A allele (0.93) was more frequent than the B allele (0.07), and the AA and AB genotypes were found at frequencies of 0.85 and 0.15, respectively. No animals with the BB genotype were found. The frequency of the A allele in our study is consistent with those reported in *B. t. indicus* cattle of the Guzerat (0.83) (Steinberg et al. 2022), Gyr (0.93) (Pessoa et al. 2022), Indu-Brasil (0.92), and Sardo Negro (0.95) breeds (Parra-Bracamonte et al. 2025). This similarity could be due to the high frequency of the A allele in the founding breeds of Sardo Negro cattle. Because the B allele is associated with improved cheesemaking performance in *B. t. taurus* cattle (Sharaz and Rabindrakumar, 2024), the frequency of the A allele increases the possibility of selecting a favorable allele and, after a genetic improvement program during several generations, increasing its prevalence, as has been done for other breeds (Barbosa et al. 2019).

## **β-LG (g.5305C > T SNP)**

Genetic variants A and B of the g.5305C > T SNP in the β-LG gene were identified in this study. The B allele (0.86) was more frequent than the A allele (0.14). The AA, AB, and BB genotypes were found with frequencies of 0.03, 0.24, and 0.74, respectively. The predominance of the B allele in this study is consistent with that reported by Parra-Bracamontes et al. (2025) in the Nellore (1.00), Brahman (0.64), Guzerat (0.76), Indu-Brasil (0.78), Gyr (0.50), and Sardo Negro (0.76) breeds. However, Badola et al. (2004) did not find significant differences in the frequencies of this polymorphism between the *B. t. indicus* and *B. t. taurus* subspecies. Selection for the B variant of the β-LG gene and the B allele of the CSN3 gene could result in a haplotype that favors cheese production when they are present in a homozygous state (Mohan et al. 2021). The availability of genotyping techniques enables the development of a selection strategy to introduce favorable alleles and increase their frequencies in this population.

## **CSN2 (c.245C > A SNP)**

In the case of the c.245C > A SNP in the CSN2 gene, the A2 and A1 alleles were found at frequencies of 0.99 and 0.01, respectively. The calculated genotype A2A2 and A1A2 frequencies were 0.98 and 0.03, respectively. No animals with

the A1A1 genotype were found. Pessoa et al. (2022) reported frequencies of the A2 allele of 0.95, 0.92, and 0.88 in the Sindhi, Guzerat, and Gyr Indica breeds, respectively. In Sardo Negro cattle, the high frequency of the A2 polymorphism could be due to the influence of *B. t. indicus* because this variant corresponds to the ancestral allele (Bisutti et al. 2022). Considering that the A2 allele is associated with healthy milk consumption (Jianqin et al. 2016), the high frequency of this genotype in Sardo Negro cattle could justify the promotion of breeding of this Mexican breed. In conclusion, the frequencies of the favorable alleles in the CAST,  $\beta$ -LG, and CSN2 genes were high in the Sardo Negro breed. Although the low frequencies of polymorphisms in the CAPN1, CSN3, and GH genes may limit the use of haplotypes with desirable phenotypic traits in this population, the available genotyping techniques could be useful tools to select relevant alleles and increase their frequencies in the Sardo Negro cattle populations in Mexico with the objective of improving their production performance.

## Declarations

## Conflict of interest

The authors declare no conflict of interests.

### Ethics approval

This study used only peripheral blood samples collected by venipuncture as part of routine veterinary procedures, in accordance with NOM-051-ZOO-1995. Sampling was performed by trained personnel using standard practices to minimize animal discomfort. No experimental interventions were conducted; therefore, ethics committee approval was not required.

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## Author contributions

Conceptualization: Aldo Francisco Galindo-Hernández and Miguel Ángel Ayala-Valdovinos; Methodology: Aldo Francisco Galindo-Hernández, Miguel Ángel Ayala-Valdovinos, and Theodor Duifhuis-Rivera; Formal analysis and investigation: Aldo Francisco Galindo-Hernández, Miguel Ayala-Valdovinos, and Clemente Lemus-Flores; Writing—original draft preparation: Aldo Francisco Galindo-Hernández, Néstor Gerardo Michel-Regalado, and Abraham Virgen-Méndez; Writing—review and editing: Aldo Francisco Galindo-Hernández, Néstor Gerardo Michel-Regalado, Abraham Virgen-Méndez, and Clemente Lemus-Flores; Funding acquisition: Miguel Ángel Ayala-Valdovinos and Theodor Duifhuis-Rivera. All the authors reviewed and approved the final version of the manuscript.

## Data availability

The datasets generated during the current study are available from the corresponding author upon reasonable request.

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Figures



**Figure 1**

Agarose gel electrophoresis of PCR–RFLP fragments for genotyping the GH gene. Lane 1: 25-bp molecular marker; Lane 2: VV homozygous control; Lane 3: LV heterozygous control; Lane 4: LL homozygous control; Lane 5: negative control; Lanes 6–12: LL genotype.

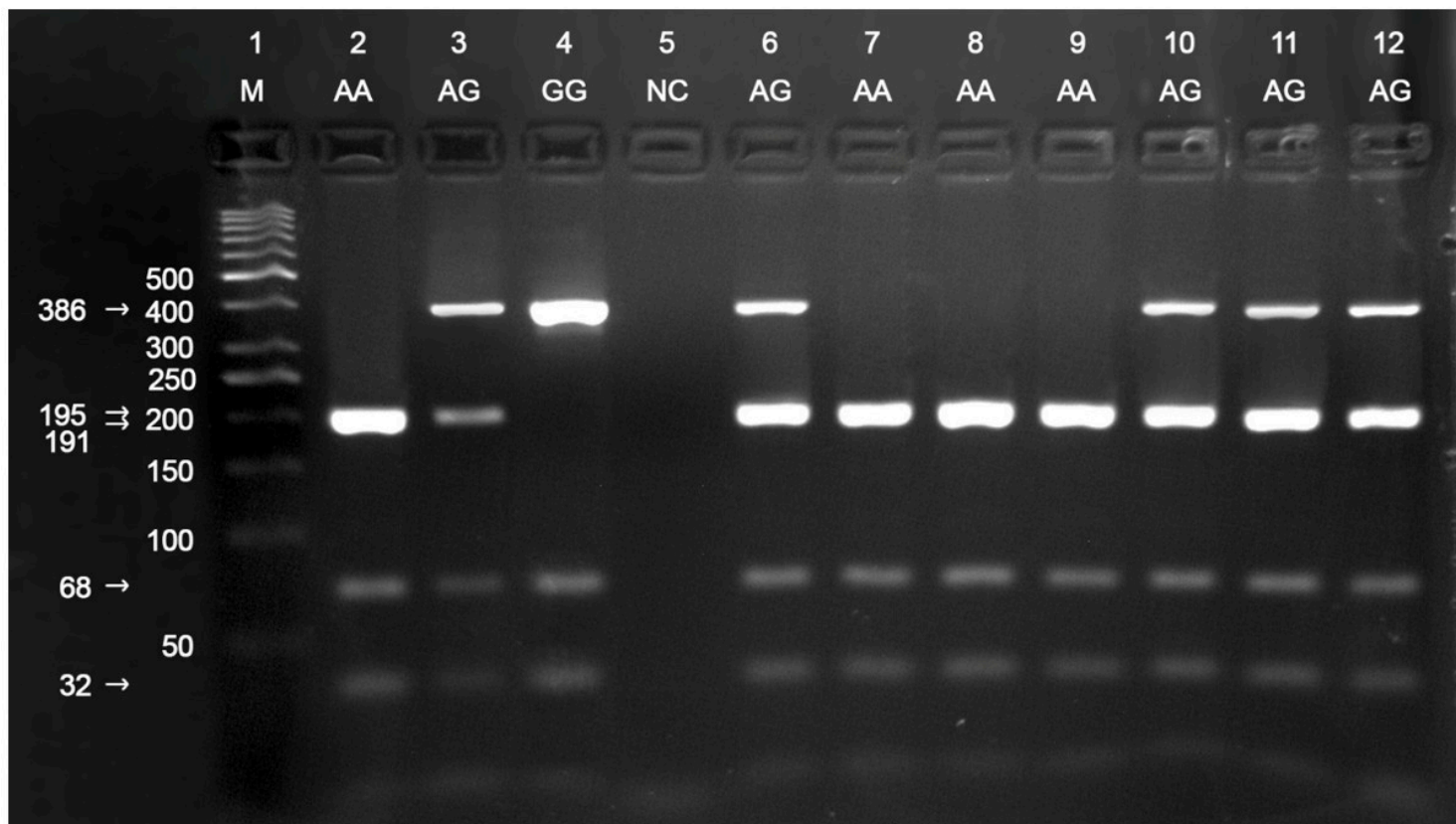


**Figure 2**

Agarose gel electrophoresis of PCR-RFLP fragments for genotyping the CAST gene. Lane 1: 50-bp molecular marker; Lane 2: AA homozygous control; Lane 3: AG heterozygous control; Lane 4: GG homozygous control; Lane 5: negative control; Lanes 6 and 10-12: AG genotype; Lines 7-9: AA genotype.

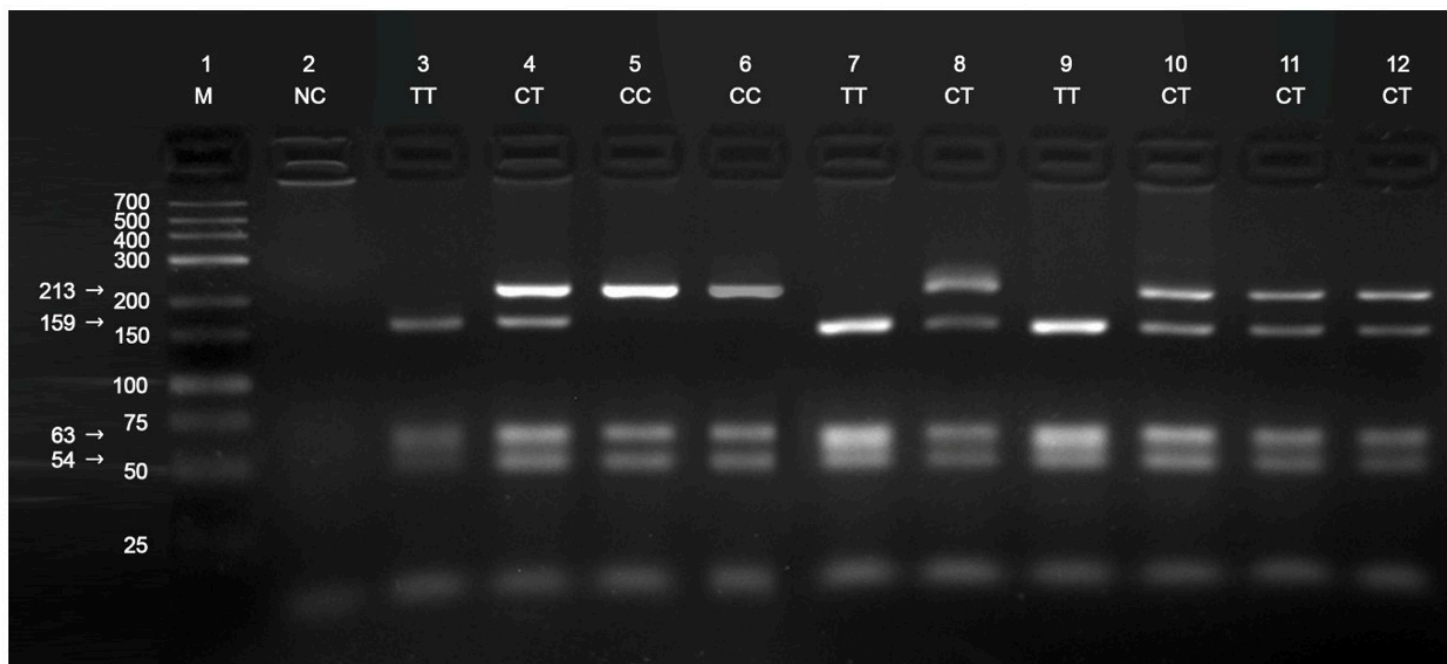


**Figure 3**

Agarose gel electrophoresis of PCR–RFLP fragments for genotyping the CAPN1 gene. Lane 1: 25-bp molecular marker; Lane 2: negative control; Lane 3: TT homozygous control; Lane 4: CT heterozygous control; Lane 5: CC homozygous control; Lane 6: CC genotype; Lines 7 and 9: TT genotype; Lines 8 and 10–12: CT genotype.

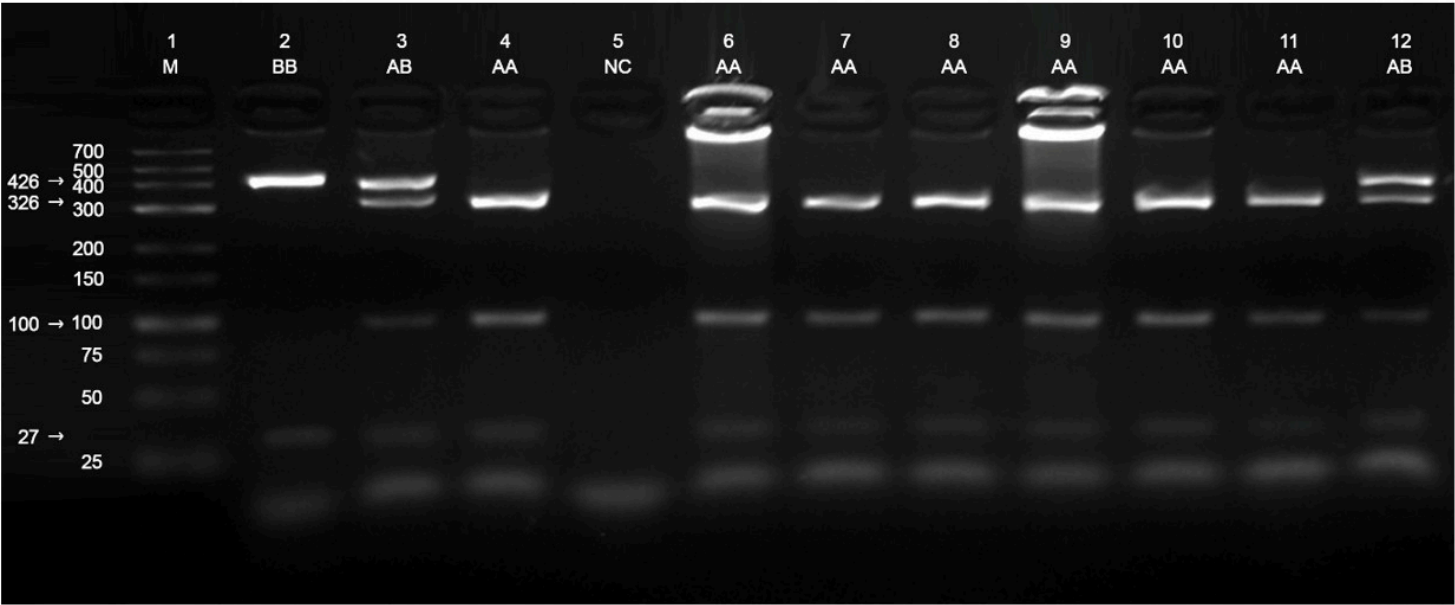


Figure 4

Agarose gel electrophoresis of PCR–RFLP fragments for genotyping the CSN3 gene. Lane 1: 25-bp molecular marker; Lane 2: BB homozygous control; Line 3: AB heterozygous control; Line 4: AA homozygous control; Lane 5: negative control; Lanes 6–11: AA genotype; Lane 12: AB genotype.

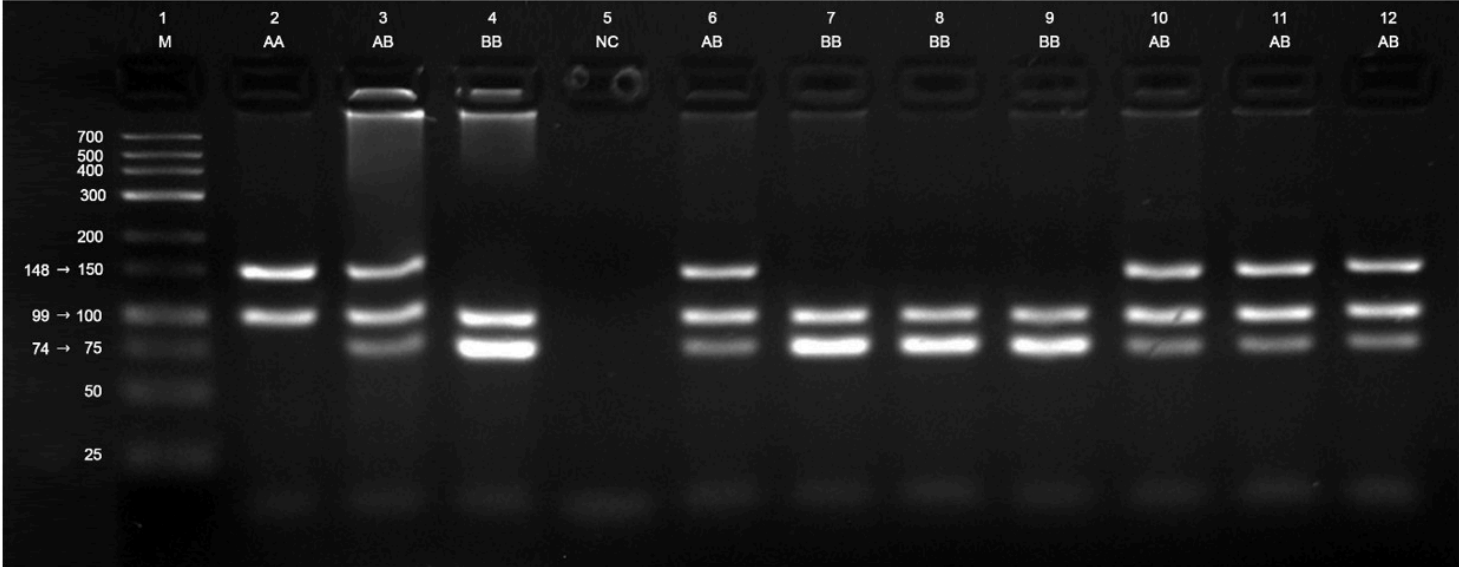


Figure 5

Agarose gel electrophoresis of PCR–RFLP fragments for genotyping the  $\beta$ -LG gene. Lane 1: 25-bp molecular marker; Lane 2: AA homozygous control; Lane 3: AB heterozygous control; Lane 4: BB homozygous control; Lane 5: negative control; Lanes 6 and 10–12: AB genotype; Lanes 7–9: BB genotype.

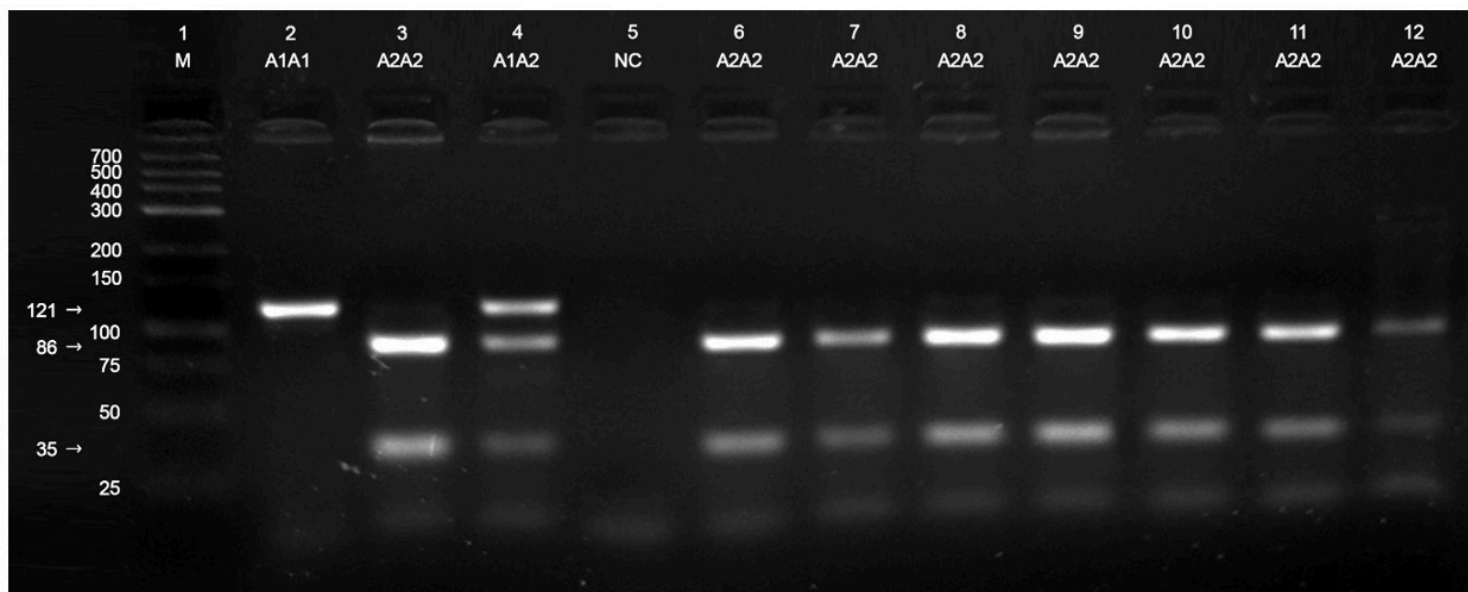


**Figure 6**

Agarose gel electrophoresis of PCR–RFLP fragments for genotyping the CSN2 gene. Lane 1: 25-bp molecular marker; Lane 2: A1A1 homozygous control; Lane 3: A2A2 homozygous control; Lane 4: A1A2 heterozygous control; Lane 5: negative control; Lanes 6–12: A2A2 genotype.