TESTING THE PRESENCE OF SNP POLYMORPHISMS IN THE 19TH INTRON OF THE CALPASTATIN (CAST) GENE ON THE ROMANIAN SPOTTED CATTLE, SIMMENTAL TYPE AND ANGUS BREED

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Abstract:

The tenderness of meat is under complex genetic determinism, being studied extensively in the last couple of years on various cattle breeds with respect to genetic variations associated with the trait. Only few of studied loci had major effect on tenderness, with calpains m and μ and calpastatin loci receiving special attention. Calpains m and μ intervene in the post-mortem proteolysis of myofibrils, being in direct relationship with their inhibitor – the calpastatin. The presence of potential Single Nucleotide Polymorphisms on intron 19 of the calpastatin gene (CAST) in mixed cattle breed (meat & milk/Romanian Spotted breed, Simmental type) and specialized meat breed (Angus) were investigated in this study. DNA samples from two cattle breeds were used to determine the SNP polymorphisms in the 552 bp fragment of the 19th intron of the CAST gene. The presence of mutations was investigated by testing the modifications occurring in BshF1 (GGCC) and RsaI (GTAC) restriction enzyme's sites. All individuals analysed have had the same electrophoretic profile suggesting that no genuine mutations occurred in both restriction enzyme's sites of CAST gene (BTA7) from the Bos taurus populations.

Key words: CAST, BshF1, RsaI, tenderness, SNP Polymorphism

INTRODUCTION

Improvement of complex traits such as the ones tied to organoleptic properties of the beef, which have low heritability and are unrepeatable, is difficult and their standardisation is even more so. In the case of cattle breeds the process is more complex, because of the long generative cycle as opposed to other species. Traditional methods of selection and breeding were based on the estimated breeding value, taking into consideration the phenotypical values of the animals or its' collateral relatives [1]. Palatability attributes of beef, valued by consumers (juiciness, tenderness, flavour, etc.) have received special attention in the last couple of years for the identification of favourable genetic variation that can be introduced in selection and breeding [2]. Studies have been focused firstly on identifying loci of candidate genes with major effect, which can be used in Marker Assisted Selection. In the last 15 years numerous polymorphisms have been tested for association, some of which are available commercially today as genetic tests (IGenity/GENE-STAR). Out of all the quantitative trait loci studied just a few have been proven to have a major effect in genetic variation. Among them, calpains loci and the calpastatin locus can be found. A major factor which contributes to meat tenderization is post mortem proteolysis of myofibrils [3; 4] and evidences of the existing direct relationship with tenderness in livestock include association with calpains and calpastatin genes variation. For these reasons many research projects proposed to introduce molecular information in breeding programmes in last ten years, to improve this parameter. They are involved in a wide range of physiological processes including muscle growth and differentiation, pathological conditions and post-mortem meat aging [5]. In the calpain proteolytic family, μ - calpain (CAPN1) is responsible for the

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breakdown of myofibril proteins, while calpastatin (CAST) inhibits μ -calpain and m calpain (CAPN2) activity and therefore regulates post-mortem proteolysis [6; 7; 8]. Therefore, increased tenderness of the beef that reaches consumers tables is the result of the two endogenous proteases, whose activity is dependent of their inhibitor. The calpastatin gene (CAST), through genetic variants, modulates the post mortem meat tenderization, being identified several genetic variants and many polymorphisms associated with a tender meat or a tougher one. These evidences are addressed specifically to SNPs polymorphisms of calpastatin and also to calpains genes. Various studies identify specific polymorphisms in CAST gene and establish association with beef tenderness in many breeds and crossbreed animals [7; 9; 10; 11; 12; 13; 14; 15]. In a recent study (2014) the CAST and CALP gene polymorphisms have been tested for association with beef tenderness in two Spanish breeds (Parda de Montana and Pirenaica). Out of 31 polymorphisms identified in CAST gene, none were found in the coding region. Five polymorphisms from CAST gene have been associated with tenderness, 7 day after slaughtering [6]. The polymorphisms are situated in the 5th and 12 introns, 7th exon and 3' UTR region. The polymorphism (g98579663A>G) of CAST gene, described for the first time by Barendse in 2002, modifies the target putative site for miRNA (bta –miR 542-5P), being associated with beef tenderness in many breeds and crossbreed animals [14; 6; 10; 17]. The A>G mutation is in the 3' UTR region (CAST_5). Also, a SNP polymorphism in the 5th intron of CAST gene (g282CNG) was associated with post mortem beef tenderness in crossed breeds *B. taurus* populations [9]. Alongside two polymorphisms, Calvo et al., in 2014, described three other polymorphisms in CAST gene, namely in CAST 2&3, in the 7th exon and in CAST _4, 12th intron, which were tested for association with tenderness in two Spanish breeds. Two polymorphisms, in the exon 7 (g98535683A>G) and on 3'UTR region (g.98579663A > G) was significant associated with a tougher meat [6]. This mutation consists in an amino acid modification Thr182Ala. However, to utilize these polymorphisms in breeding programs it is mandatory that each of them is tested in different populations. In the present work we tested for possible presence of SNP polymorphisms in 19th intron of the CAST gene on Romanian Spotted cattle, Simmental type, and comparative with the Angus breed.

MATERIAL AND METHOD

DNA samples:

35 animals from different populations were used to test the presence of putative genetic polymorphisms. (25 animals of Romanian Spotted cattle, Simmental type – a mixed breed for milk and meat and 10 animals of Angus meat breed). The blood samples from which DNA was extracted come from animals included in different experiments conducted between years 2016-2018 at Laboratory of Molecular genetics and Biotechnologies of our University. *Genomic DNA extraction from blood* -200 μl of blood samples collected on K_2EDTA, from each animal, were subjected to extraction with Quick DNA Microprep Plus Kit following the manufacturer's instructions (BioZyme). The DNA samples were then analysed on Spectrophotometer NanoDrop ND1000 to determine the quantity and quality of the DNA. All samples analysed had optimum purity, ranging between 1.8 and 2 and the quantity, ranging between 50-78 ng DNA/μl.

Primer design and PCR amplification

Nucleotide structure of the intronic region of CAST gene, taken into account in this study was identified in the NCBI database. The polymorphism study was conducted initially using the genetic database to identify the DNA fragment to be amplified. The region selected to be amplified by PCR was identified based on Accession Number-AH014256.2 of CAST gene sequence that corresponds to the 19th intron and a small previous region (of 19 nucleotides). For this study, the previous regions with polymorphisms associated with tenderness were excluded [18; 9; 19; 11; 6]. The fragment to be amplified by PCR had 552 bp and the primers design to amplify this fragment of the CAST gene was realised with Primer3 Premier Software. Their sequence is presented in Table 1.

Table 1 The 5'-3' primer's structure for amplification of 552 bp fragment of the 19th intron of CAST gene (BTA7)

	Primer structure	Ta
Forward	5' ATCCAGAAGACGGAAAGCCT 3'	58ºC
Reverse	5' CTCACGATCCTCTTC TTTGG 3'	

PCR Amplification

All the DNA samples were amplified by PCR in a 25 µl final volume with following composition: 5x FirePol Master Mix (BioZyme) – 5µl; 0.6 µl of each (forward and reverse) primer (from 10pmol/µl solution), DNA template - 2µl and H_2O -16.8 µl. The PCR thermal cycling conditions were: 95ºC (5 min) – 1 cycle; 35 x 94ºC – (30 s); 58ºC (45 s) and 72ºC (45 s); and final extension at 72 ºC for 8 min, maintaining 4ºC thereafter. The PCR reactions were performed in an Eppendorf MasterCycler thermocycler (Eppendorf, Germany). The amplimers of 552 bp were than subjected to digestion with *Bsh*FI and *Rsa*I restriction enzymes. The possible SNP mutations in the GGCC site of *Bsh*F1 restriction enzyme and also in the GTAC site of *Rsa*I restriction enzyme site were analysed.

RFLP analysis

The PCR products were submitted to digestion with two restriction enzymes to identify the possible causative mutations abolishing the restriction sites. The possible SNP mutations were searched in the positions 149 and 235 bp, with *Bsh*F1 restriction enzyme (GGCC) and also in the positions 242, 314 and 491 bp, which correspond to *Rsa*I restriction enzyme site (GTAC) in the analysed fragment. The following protocol for enzymatic digestion was applied. The reaction mixture was set for a final volume of 20 µl for each restriction experiment, with 10 µl PCR product, 2 µl restriction enzyme's buffer, 0.6 µl restriction enzyme (for each digestion experiment) and 7.4 µl H_2O. The samples were incubated at 37ºC for 3 h and the digestion products were submitted to electrophoresis in a 2.5 % agarose gel. After restriction analysis performed on the two breeds all electrophoretic profiles reveal no SNP mutations, being all monomorphic. The presence of restriction sites in the 552 bp fragment, corresponding to the two enzymes, was confirmed by expected fragments after digestion. The *Bsh*F1 enzyme has two restriction sites in the fragment (position 149 and 235 bp) and *Rsa*I has three restriction sites in the same fragment (positions 242, 314 and 491 bp).

RESULTS AND DISCUSSIONS

After digestion of 552 bp fragment with *Bsh*F1 restriction enzyme, 3 fragments of 316, 149 and 86 bp were obtained for all samples (Fig. 1). The monomorphic profile of each sample denotes that no mutation was occurred in GGCC restriction site of *Bsh*F1 enzyme. For *Rsa*I restriction enzyme, 3 fragments of 314 bp, 177 bp and 60 bp respectively were obtained for each sample (Fig. 2). These profiles show also that no mutations have occurred in the *Rsa*I restriction site, and all individuals have the same profile.

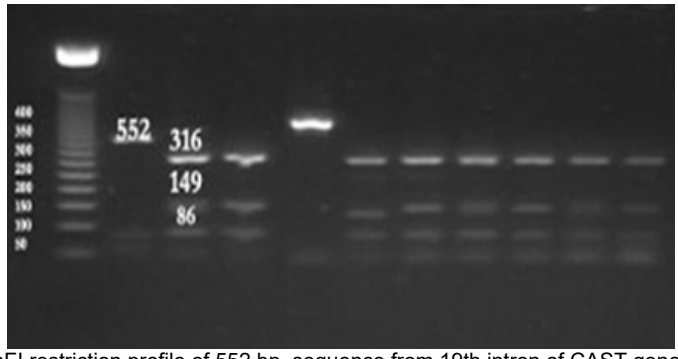

Fig. 1 The BshFI restriction profile of 552 bp sequence from 19th intron of CAST gene (line 1- 50 bp DNA ladder; line 2 PCR product, and CAST/ BshFI fragments of 316, 149 and 86 bp from Romanian spotted cattle, Simmental type and Angus individuals

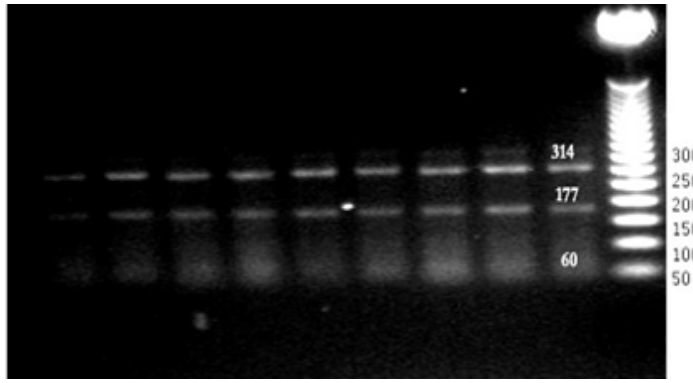

Fig. 2 The BshFI restriction profile of 552 bp sequence from 19th intron of CAST gene (line 10 - 50 bp DNA ladder; line 1-9 restricted PCR products of 19th intron of CAST gene from Romanian spotted cattle, Simmental type and Angus individuals (314, 177 and 60 bp)

In the literature, three polymorphisms on the CAST gene are well documented. All of these were associated with increased tenderness of the beef in various breeds and crossed breeds. These are BTA7 g.98533962C>G in the 5th intron [9], BTA7 g.98535683 A>G in the 7th exon and mutation g.98579663A>G in the exon 30/3'-UTR region [18; 10]. Among the studied breeds are: Angus, Limousin, Charolais, Simmental [9]; Brahman Belmond Red, Angus, Hereford [18], Jersey x Limousin, Angus x Hereford cross - cattle [20]; Chinese commercial cattle [11], Brahman, Nellore, Angus and crossed breeds: Angus x Nellore, Rubia Galega x Nellore, Canchim, Brown Swiss x Nellore [10], Hereford, Charolais, Gelbviech and Simmental [14], Parda de Montana and Pirenaica [6], Hanwoo breed [21]. Another study analysed 28 SNPs, grouped in 3 LD blocks on the CAST gene (BTA7). The three blocks lie between the 3rd intron and 9th exon (LD block 1); intron 12 and exons 20 and 22 (LD block 2) and finally, in the third block between intron 25 to exon 31 (including a segment of 3' UTR region). Out of the 15 initially identified polymorphisms in the 3rd block, 4 of them were associated with tenderness on the Angus-Brahman hybrids [22].

CONCLUSIONS

In conclusion, the extensive study of CAST gene polymorphisms suggests the still unexplored potential of genetic determinism for establishing associations with meat tenderness in different breeds or crossed breeds. A recent study in three French meat breeds has also indicated that distinct effects of the calpastatin markers are attributed to the breed and cannot be extended to all Bos taurus breeds [17]. Another conclusion about meat tenderness is drawn by Pintos and Corva (2011) in marker association studies for meat tenderness and growth traits on Angus breed. They suggest that any recommendations to improve beef tenderness, using molecular markers in the CAPN1 and CAST genes, should take into account the fact that there could be a correlated response in growth traits. Consequently, the further exploration of the genetic polymorphisms in the calpastatin and calpain genes, in the Romanian spotted cattle - Simmental type, remains open, with the potential to identify new genetic polymorphisms.

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