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Nucleotide sequence and variations of the bovine myocyte enhancer factor 2C (*MEF2C*) gene promoter in *Bos Taurus* cattle

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Abstract Myocyte Enhancer Factor 2 (*MEF2*) proteins are a small family of transcription factors that play pivotal role in morphogenesis and myogenesis of skeletal, cardiac, and smooth muscle cells. In vertebrates, there are four *MEF2* genes, referred to as *MEF2A, -B, -C,* and *-D*, that are located on different chromosomes. After birth *MEF2A*, *MEF2B*, *MEF2D* transcriptions are expressed ubiquitously, whereas *MEF2C* transcripts are restricted to skeletal muscle, brain, and spleen. In this study, on the basis of the sequences of the bovine chromosome 7 genomic contig, available in the *GenBank* database, sets of PCR primers were designed and to amplify the bovine *MEF2C* gene promoter region, exon 1 (*5′UTR*) and part sequence of the intron 1. Seven overlapping fragments of the bovine *MEF2C* gene were amplified and then sequenced. Altogether, these fragments were composed in the 3,120-bp sequence which was deposited in the *GenBank* database under accession no. GU211007. The sequence fragment included the putative site of the promoter region and transcription start of the exon 1. The sequence analysis of these fragments in individual animals representing different *Bos taurus* breeds revealed four variations in promoter region: *g.-1606C>T*, g.*-1336_-1335DelG*, g.*-818C>T*, g.*-613_-612DelA* and four SNPs within intron 1: g.*2711A>G*, g. *2913A>G*, g.*2962G>T* and g.*3014A>G*. No polymorphism was found within sequence of the exon 1 (*5′UTR*). These polymorphisms were identified for first time using these sequences and were confirmed by *RFLP* or *MSSCP* methods.

Keywords *MEF2* family · *MEF2C* gene · Polymorphism · MSSCP · RFLP · Promoter · *5′UTR*

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Introduction

The process of differentiation from mesodermal precursor cells to myoblasts has led to the discovery of a variety of tissue-specific factors that regulate muscle gene expression. The myogenic basic helix-loop-helix proteins, including MyoD, MYOG, MYF5 and MRF4 are one class of identified factors.

Recently, a family of transcription factors, the myocyte enhancer factor-2 (MEF2) family, has been shown to play a pivotal role in morphogenesis and myogenesis of skeletal, cardiac and smooth muscle cells. In vertebrates, there are four *MEF2* genes, referred to as *MEF2A*, *-B*, *-C* and *-D* that are located on different chromosomes [1]. There is a single *MEF2* gene in each of the genomes of *Drosophila* and the intron–exon organization of the *MEF2* genes from vertebrates and *Drosophila* is identical within the conserved regions of the genes, consistent with the notion that they evolved from a common ancestral *MEF2* gene [2]. Myocyte enhancer factor 2C is selectively expressed in differentiated myocytes and activates nearly all skeletal and cardiac muscle genes by binding a conserved A/T-rich DNA sequence in the control region of them [3]. *MEF2* family share homology within the MADS box domain (56 amino acids), which mediated DNA binding and dimerization. An additional *MEF2* domain (29 amino acids), adjacent to the MADS box, is also highly conserved among

the *MEF2* proteins but is absent from other MADS box proteins [4].

Expression of the *MEF2A*, -C and -D genes marks early myogenic lineages during mouse and frog embryogenesis. *MEF2*C is the first member of the family to be expressed in the mouse with transcripts appearing in the precardiac mesoderm at day 7.5 postcoitum. In skeletal muscle cells, *MEF*2C is expressed within the somite myotome beginning at about 9.0 days postcoitus and *Mef*2A and -D are expressed immediately thereafter. After birth *MEF2*A, *MEF*2B and *MEF*2D transcripts are expressed ubiquitously. *MEF*2C transcripts are restricted to skeletal muscle, brain and spleen [5]. Despite all the available information concerning *MEF2* genes, little is known about how this gene is regulated at the transcriptional and translational level, especially in bovine. Because of the economic importance of the bovine species to the livestock industry, it appears clearly essential to clarify some of the factors related to MEF2 genes expression.

The present study was conducted to identify new polymorphisms in the promoter region of the bovine *MEF2C* gene, and to determine the allele distribution in different cattle breeds.

Materials and methods

Animals and DNA samples

Blood samples were collected from unrelated bulls of different breeds: Aberdeen Angus (8), Charolaise (10), Hereford (34), Limousine (27), Simmental (9), Polish Friesian (136) and Polish Red (47). Approximately 10 ml blood was withdrawn from each animal to test tubes containing K_2EDTA by an authorised veterinarian. DNA was isolated from blood by the method of Kanai, Furii, Saito & Tokoyama [6]. All procedures involving animals were performed in accordance with the Guiding Principals for the Care and Use of Research Animals and were approved by the Local Ethics Commission (Permission No. 3/2005).

Identification and analysis of polymorphism

Basing on the sequences of the bovine chromosome 7 genomic contig (*GenBank acession number*: NW_001495 275) available in the GenBank database and using the Apollo Genome software (http://www.dhgp.org/current/install.html) were determined sequence two fragments: (1) the 5′noncoding region encompassed site of the promoter region; (2) the exon 1 (*5′UTR*) and part sequence of the intron 1. Seven pairs of primers were designed using the Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) to amplify overlapping fragments of the bovine *MEF2C* gene (Table 1). The PCR reaction was performed in final a volume of 10 µl containing 1.0 µl (30 ng/µl) genomic DNA, 0.5 µl of each primer (10 pmol/µl), 5.1 µl HotStarTag Master Mix (Qiagen, Germany), and 2.9 µl water PCR reagent. The following PCR cycle condition was used: an initial denaturation at 95°C for 15 min; 35 cycles of 95°C for 60 s, 53–61°C (depends on the primer pair used) for 40 s and 72°C for 60–80 s and final elongation at 72°C for 10 min. The yield and specificity of PCR products were evaluated after electrophoresis in 2% agarose gel (Sigma-Aldrich, Germany) with ethidium bromide. Then the PCR products were purified with the GenElute PCR DNA Purification Kit (Sigma-Aldrich,

Table 1 Primers and annealing temperatures used for PCR-SSCP analysis of the *MEF2C* gene

Locus	Primer sequences	Mutation[a]	Region	Position	Length	Tm (°C)
P1	F: ACTGTTATTGTGTACTCTTGTACATCC R: TGACTTCACTTATCCTGAAGAGTTT	*g.-1606C>T*	Promoter	69–451	382	57.0
P2	F: AGGGAGAGGAGGTGCTCAAT R: CCGGCATTTGAAATCTTGAC	g.*-1336_1335DelG*	Promoter	277–663	386	59.5
P3	F: CTGCCGCCTCGGTAAATA R: AGCCAGCCAGCGGGAAAGTC	–	Promoter	588–992	404	61.0
P4	F: TTAGAAGGACTTTCCCGCTG R: ATGATAATTGCATCTTGGGT	g.*-818C>T*	Promoter	966–1230	264	54.0
P5	F: AGATTGATTCACCCAAATG R: GCCTTTGAAACATAGCGTC	g.*-613_-612DelA*	Promoter	1200–1694	474	57.0
P6	F: CGCCTTTTTGACGCTATGTT R: CCCGGCTATTTCTATTTCC	–	Exon 1 (5′UTR)	1678–2298	620	61.0
P7	F: CCGGGGTATTGTCTCTGAAA R: GCCATATTCTGGGAAGGACA	*g.2711A>G; g.2913A>G; g.2962G>T; g.3014A>G*	Intron 1	2294–3120	826	60.0

[a] Polymorphisms in promoter region numbered relative to the transcription start site in exon 1

Germany) and sequenced in an ABJ377 sequencer (Applied Biosystem, CA, USA). The sequencing was done at the Polish Academy of Science Institute of Biochemistry and Biophysics, Warsaw. The amplified fragments of the *MEF2*C gene were sequenced from twenty individual animals belonging to four cattle breeds: Fresian (5), Polish Red (5), Hereford (5) and Limousine (5). Nucleotide sequence alignments were carried out using the Sequencher 4.8 Demo software (www.genecodes.com).

Restriction map of the amplified *MEF2*C gene fragments was done using Nebcutter software (http://tools.neb.com/NEBcutter2/index.php).

Identified polymorphisms in promoter region were confirmed by *RFLP* or *MSSCP* methods. The 382-bp PCR product including P1 fragment was digested for 3 h with 5 U of *Bsr*I (New England Biolabs) restriction endonucleases. The restriction products were separated by electrophoresis in 3% agarose gel (Sigma-Aldrich, Munich, Germany) with ethidium bromide in 1× TBE buffer. Gels was visualised and documented by the Molecular Imager System FX (BioRad, CA, USA).

However, PCR-multitemperature single-strand conformation polymorphism technique was using for PCR products amplified including P2, P4 and P5 fragments. Samples of DNA were prepared according to procedure for *MSSCP* Starter Kit (Kucharczyk Co., Poland). 1 µl of PCR products were mixed with 5 µl denaturing A buffer. This mixture was heated for 10 min at 98°C and chilled immediately on ice and was added to the samples 2 µl of denaturing B buffer. The mixture was loaded onto a 9% PAGE (polyacrylamide gel, 249 mm × 197 mm) containing 15% glycerol. Electrophoresis was carried out in 0.5× TBE buffer in a DNA Pointer System (Kucharczyk Co., Poland) with 40 W constant power. The gel temperatures were as follows: 35°C for 350 Vh, 15°C for 350 Vh and 5°C for 350 Vh. The total time of electrophoresis was about 70 min. After electrophoresis the gels were silver stained for 30 min using the Silver Stain Kit (Kucharczyk Co., Poland) and then scanned and documented by the Molecular Imager System FX (BioRad, CA, USA).

Genotype and allele frequencies and the Hardy–Weinberg balance were calculated with the use of POPGENE ver. 1.31 software (http://www.ualberta.ca/~fyech). The significance of differences in allele and genotype frequencies was compared with the use of the χ^2 test.

Computer analysis

The nucleotide sequence of the promoter region of the *MEF2C* gene was analyzed for the presence of putative TF-factor binding sites. Sequences with 100% identity to TF-binding sites were searched in TESS software (Schug, J. & Overton, Ch.G.; http://www.cbil.upenn.edu/tess).

Results and discussion

We amplified and then sequenced several overlapping fragments of the noncoding and coding region of the bovine *MEF2*C gene including promoter region, all sequence of the exon 1 (5′UTR) and part of sequence of the intron 1 (Fig. 1). Altogether, these fragments were composed into a 3,120-bp sequence which was deposited in the *GenBank* database under accession no GU211007. In this study DNA sequencing method were used to detect genetic variations of the *MEF2C* gene in seven different cattle breeds. Sequence analysis revealed four polymorphic sites (*g.-1606C>T*, g.*-1336_-1335DelG*, g.*-818C>T*, g.*-613_-612DelA*) in promoter region and four SNPs in intron 1 (g.*2711A>G*, g. *2913A>G*, g.*2962G>T* and g.*3014A>G*) (Fig. 2A, B). Comparison of the restriction maps of the both *MEF2*C gene variants revealed that the only substitution *g.-1606C>T* was recognized by *Bsr*I, nuclease, thus enabling the *RFLP* analysis of the gene polymorphism. SNPs identified within intron 1 were not analyzed by any method. Moreover, for three remaining mutations (g.*-1336_-1335DelG*, g.*-818C>T*, g.*-613_-612DelA*) no any restriction enzyme recognition site were found, and therefore for these mutations we used *MSSCP* technique (Fig. 3a). Only two genotypes were identified in panel of bulls belonging to seven breeds by using MSSCP method in the relation to variations: g.*-1336_-1335DelG*, g.*-818C>T* and g.*-613_-612DelA*. The heterozygotes G/– (g.*-1336_-1335DelG*), T/C (g.*-818C>T*) and A/– (g.*-613_-612DelA*) appeared very rare and were found only in Fresian and Polish Red breeds; all the three *MSSCP* loci only one homozygous genotype was found (Table 2).

Using the *RFLP* method, the SNP *g.-1606C>T* was genotyped in 271 bulls from different breeds. After digestion with *Bsr*I nuclease, three CC (129, 122, 117-bp), TT (246, 122-bp) and CT (246, 129, 122, 117-bp) genotypes were identified in the all breeds (Fig. 3b).

The results of χ^2 test for different genotypes frequencies were shown in Table 3. No significant differences in genotype frequencies were found within the breed group from the Hardy–Weinberg proportions. The results showed that the occurrence of different genotypes and alleles might vary between breeds. The frequency of allele C varied from 0.654 in Fresian to 0.044 in Hereford, while the frequency of allele T was within the range of 0.346–0.956, respectively. In beef cattle breeds the TT genotype was predominant, but Fresian breed showed the greatest frequency of CC genotype. The CC genotype was not observed in Simental, Charolaise, Hereford and Angus bulls. There was only one CC animal found in the Limousine breed. Probably, it was caused small number of animals within analyzed group of cattle.

Fig. 1 Nucleotide sequence of the fragments of the bovine *MEF2*C gene including eight polymorphic sites. The SNPs are shown in *brackets* and *bold letters*; exon 1 is marked in *bold letters*

Many important traits of domestic animals are controlled by multiple genes and complex gene interaction. The study of candidate genes can be useful to determine whether specific genes are related to the economic traits. The MEF2 proteins are involved in regulation of many muscle specific genes and play active roles in myogenesis, proliferation and differentiation [7]. However, much of the available information about these genes was taken from studies on humans and might not be directly applicable to poultry.

It is known that, from molecular markers that polymorphism at the DNA level is key players in animal genetics. Allelic variation in the regulatory and structural regions of these genes may effect the gene expression or the acid sequence of product and ultimately impact on beef quality traits.

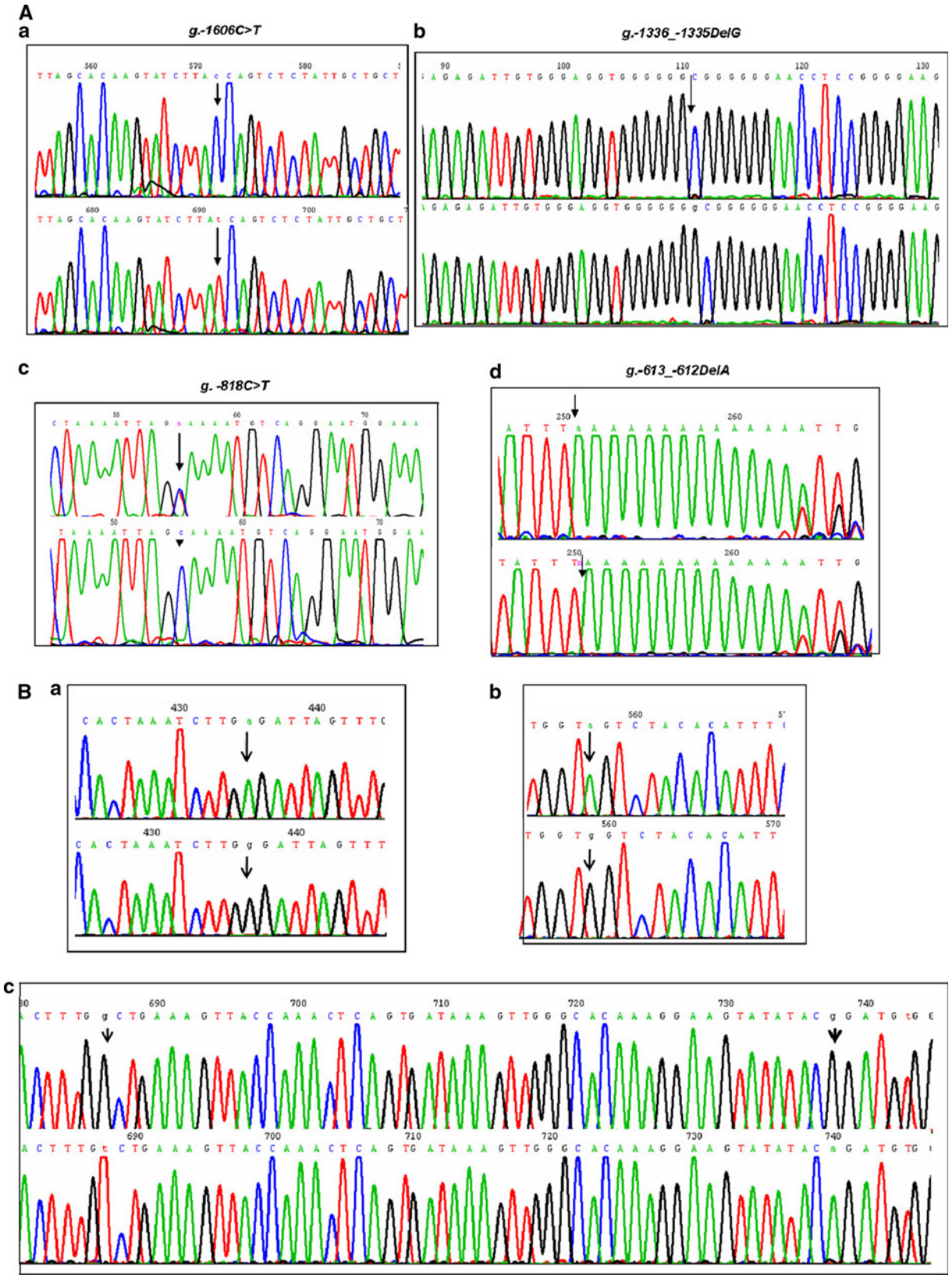

Fig. 2 Sequence analysis of polymorphic regions of the bovine *MEF2*C gene. Mutation sites are indicated by *arrows* (**A**). Mutations within the promoter region: **a** *g.-1606C>T*; **b** g.*-1336_-1335DelG*; **c** g.*-818C>T*; **d** g.*-613_-612DelA*. **B** SNPs within intron 1. **a** g.*2711 A>G*; **b** *2913A>G*; **c** g.*2962G>T*; **d** g.*3014A>G*

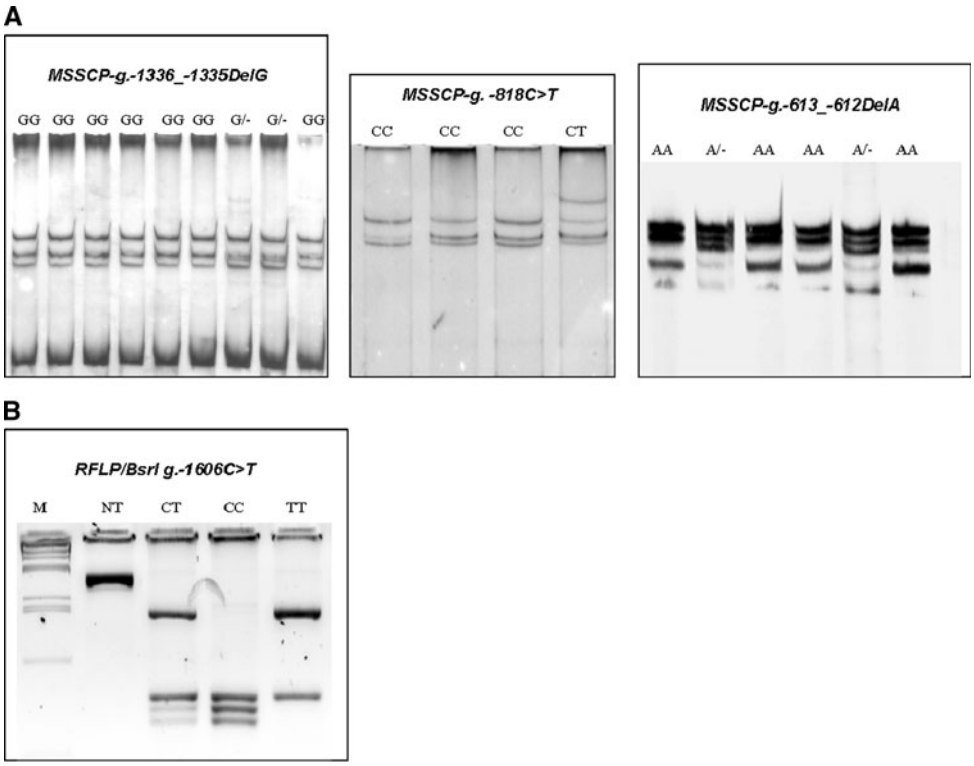

Fig. 3 RFLP/MSSCP genotyping of the two nucleotide substitutions and two deletions in promoter of the bovine *MEF2*C gene. **a** *RFLP* genotyping of the *g.-1606C>T* with *Bsr*I nuclease; **b** MSSCP genotyping of the *DelG* at position *-1336_-1335*; **C** MSSCP genotyping of the C/T transition at position *-818*; D. MSSCP genotyping of the *DelA* at position *-613_-612*

Table 2 Genotypic frequencies of the SNPs identified in the promoter region of the *MEF2C* gene in Polish Fresian and Polish Red breeds

Locus	Genotype	Polish Fresian	Polish Red	Hereford	Charolaise	Simmental	Abeerden Angus	Limousine
g.*-1336_1335DelG*	GG	134	47	34	10	9	8	27
	–/–	0	0	0	0	0	0	0
	G/–	2	0	0	0	0	0	0
g.*-818C>T*	CC	136	43	34	10	9	8	27
	CT	0	0	0	0	0	0	0
	TT	0	4	0	0	0	0	0
g.*-613_-612DelA*	AA	134	47	34	10	9	8	27
	–/–	0	0	0	0	0	0	0
	A/–	2	0	0	0	0	0	0

The primary mode of regulation of *MEF2* expression during embryogenesis appears to be at the level of mRNA accumulation. In vertebrates *MEF2* transcripts are highly enriched in developing muscle cell lineages during embryogenesis. In skeletal muscle cells in culture *MEF2D* has been reported to be expressed in proliferating myoblasts prior to the onset of differentiation. *MEF2A* protein appears as cells enter the differentiation pathway, and *MEF2C* is expressed late in the differentiation program. The significance of these different temporal patterns of *MEF2* expression is unclear. In the mouse and chick, *MEF2C* is the first the *MEF2* genes to be expressed, with transcripts appearing initially in mesodermal precursors that give rise to the heart [8]. In adult tissues, transcripts for *MEF2C* are restricted to skeletal muscle, brain, and spleen, whereas transcripts for the other *MEF2* factors are expressed more ubiquitously [9]. Analysis using the TESS software showed that allele C creates a SF1, GATA-1, while the G allele alternatively creates a GATA-2, GATA-3, TBP, NF-E1b binding sites, which are perfectly localized with the C/T transition at position −1,606 bp and may have an influence on the expression level of the *MEF2*C gene. In the present study genetic variations in bovine *MEF2C* gene were identified for the first time in this study.

Table 3 Genotypic (%) and allelic frequencies (%) and value of χ^2 test of bovine *MEF2C* gene

Breed	Genotype	Number of animals	Genotypic frequency (%)	Allele	Allelic frequency (%)	χ^2 (*P* value)
Polish Fresian	CC	54	39.7	C	65.4	2.59 (0.1077)
	CT	70	51.5	T	34.6	
	TT	12	8.8			
Polish Red	CC	10	21.3	C	41.5	1.32 (0.2512)
	CT	19	40.4	T	58.5	
	TT	18	38.3			
Hereford	CC	–	–	C	4.4	0.07 (0.7878)
	CT	3	8.8	T	95.6	
	TT	31	81.2			
Charolaise	CC	–	–	C	30	1.84 (0.1753)
	CT	6	60.0	T	70	
	TT	4	40.0			
Simmental	CC	–	–	C	5.6	0.03 (0.8599)
	CT	1	11.1	T	94.4	
	TT	8	88.9			
Abeerden Angus	CC	–	–	C	18.8	0.43 (0.5139)
	CT	3	37.5	T	81.3	
	TT	5	62.5			
Limousine	CC	1	3.7	C	18.5	0.01 (0.9247)
	CT	8	29.6	T	81.5	
	TT	18	66.7			

The numbers of variations identified in this region were low, suggesting that bovine *MEF2C* gene is lowly polymorphic. The two substitutions and two *InDel*s were found upstream the putative transcription start of the exon 1. That only *g.-1606C>T* substitution was recognized with *Bsr*I nuclease and analyzed by *RFLP* technique. Three remaining SNPs were found only in two Polish breeds, and frequency of heterozygotes was low. The only one mutation the promoter region appeared to have a high frequency in all the genotypes. A minimal frequency of the allele C in beef breed was caused small number of animals which were analyzed. No polymorphism was found in the exon 1, but four substitutions were identified in sequence including part of the intron 1.

Similar polymorphisms have not previously been reported in other species, and its functional significance is unknown. Recently, Zhou et al. [10] described three new polymorphisms in the chicken *MEFA* gene which were associated with carcass traits in chicken. Authors suggest that the *MEF2A* gene may be a potential marker affecting muscle trait of chickens.

In summary, newly identified polymorphism (*RFLP*/*Bsr*I) in the *MEF2C* gene could be potential genetic markers for carcass and meat quality traits in cattle. The results of this study may be useful in further research supporting associations between polymorphism in the promoter region of the bovine *MEF2C* gene, gene expression and cattle performance traits.

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