

# PYROSEQUENCING: ITS POTENTIAL AND LIMITATIONS IN DIAGNOSIS OF INHERITED DISEASES IN CATTLE

**N. A. Kirsanova<sup>1</sup>, A. V. Sukhoedova<sup>2</sup>, M. A. Pleskacheva<sup>3</sup>, I. V. Soltynskaya<sup>4</sup>, I. A. Timofeeva<sup>5</sup>, O. V. Prasolova<sup>6</sup>, E. V. Krylova<sup>7</sup>**

<sup>1</sup> Researcher, FGBU "VGNKI", Moscow, Russian Federation, e-mail: [n.kirsanova@vgnki.ru](mailto:n.kirsanova@vgnki.ru); ORCID ID 0000-0002-1666-3058

<sup>2</sup> Junior Researcher, FGBU "VGNKI", Moscow, Russian Federation, e-mail: [a.sukhoedova@vgnki.ru](mailto:a.sukhoedova@vgnki.ru); ORCID ID 0000-0002-7552-4816

<sup>3</sup> Laboratory Research Assistant, FGBU "VGNKI", Moscow, Russian Federation, e-mail: [pleskacheva@vgnki.ru](mailto:pleskacheva@vgnki.ru); ORCID ID 0000-0002-6721-2812

<sup>4</sup> Interim Head of Department, FGBU "VGNKI", Moscow, Russian Federation, e-mail: [i.soltynskaya@vgnki.ru](mailto:i.soltynskaya@vgnki.ru); ORCID ID 0000-0002-6274-5265

<sup>5</sup> Researcher, FGBU "VGNKI", Moscow, Russian Federation, e-mail: [i.timofeeva@vgnki.ru](mailto:i.timofeeva@vgnki.ru); ORCID ID 0000-0002-6996-1817

<sup>6</sup> Senior Researcher, Candidate of Science (Veterinary Medicine), FGBU "VGNKI", Moscow, Russian Federation, e-mail: [o.prasolova@vgnki.ru](mailto:o.prasolova@vgnki.ru); ORCID ID 0000-0001-8924-2273

<sup>7</sup> Leading Researcher, Candidate of Science (Biology), FGBU "VGNKI", Moscow, Russian Federation, e-mail: [e.krylova@vgnki.ru](mailto:e.krylova@vgnki.ru); ORCID ID 0000-0003-0080-0158

## SUMMARY

A large number of different groups of livestock diseases in which heredity plays a significant role are currently reported. In particular, more than 500 genetically determined morphological and functional disorders have been detected in cattle; for 150 of them, specific mutations are known. The sale of bovine genetic materials is associated with the spread of various diseases caused by mutations that occur in the prominent representatives of breeds. The abundance of lethal mutations in the populations requires a broader application of molecular diagnostic methods for detection of monogenic hereditary diseases. DNA pyrosequencing, being the most convenient technique for the rapid diagnosis of single nucleotide mutations in the bovine genome that are located in the regions with known nucleotide sequence, has a potential for meeting this need. Pyrosequencing-based methods for identification of the most common significant mutations in Holstein, Simmental, Brown Swiss and Aberdeen Angus cattle were developed, validated and approved at the FGBU "VGNKI". Such mutations are associated with leukocyte adhesion deficiency, complex vertebral malformation, uridine monophosphate synthetase deficiency, citrullinemia, spinal muscular atrophy, spinal cord demyelination, Brown Swiss haplotype 2, Weaver syndrome, developmental duplications,  $\alpha$ -mannosidosis, dwarfism, bovine male subfertility, thrombocytopathy, arachnomelia syndrome, hypozincemia-like syndrome. These methods are intended to test semen in the breeding centres, scientific laboratories working in the field of biotechnology and animal reproduction, livestock reproduction centres. The use of the proposed genetic tests to detect mutant alleles, as well as reduced use of mutation-bearing animals in stock breeding will allow minimizing the occurrence of inherited diseases and thus improving the gene pool of cattle in the country.

**Key words:** pyrosequencing, inherited diseases, genetic abnormalities, polymorphism, cattle.

## INTRODUCTION

Intensive use of the world genetic pool and reproduction biotechnologies (artificial insemination, embryo transplantation) allows significant genetic improvement aimed at animal productivity increase by getting offspring from breeding animals being the breed leaders. However, such approach to selection for desirable characteristics of productivity in cattle breeds has resulted in accumulation of the mutations associated with various pathologies [3, 6]. This reduces reproductive performance and fertility, newborn and young animal viability, duration of animal practical use resulting in significant economic losses. Therefore, development of methods for early detection of harbored mutations in breeding animals and rejection of genetically abnormal semen straws

are of current importance. Animals carrying mutations associated with different diseases can be detected only with molecular and genetic methods.

As a rule, monogenic genetic disorders can be phenotypically identified only in homozygous recessive carriers at late postembryonic development stage or in early postnatal period. Such carriers are often non-viable. In heterozygous carriers such mutations are not phenotypically apparent. DNA testing of replacement young animals will allow latent carriers detection and inherited disease spread prevention in cattle populations. Alleles undesirable for the population can be rapidly eliminated within one generation and selection process can be significantly accelerated.

More than 500 genetically determined morphological and functional disorders have been detected in cattle; 150 of them are known to be associated with specific mutations [9, 11]. Among six the most common breeds the highest number of such abnormalities were recorded in Holstein breed followed by Friesian, Black-and-White, Simmental, Brown Swiss and Ayrshire breeds (45, 32, 26, 24, 20 and 19 abnormalities, respectively). Genetic nature of the majority of cattle inherited diseases is associated with point mutations that are commonly single-nucleotide polymorphisms (SNP) in specific gene sites. A wide range of molecular-biological techniques based on polymerase chain reaction (PCR) is used for SNP detection in the genome [1]. The most common methods are as follows: restriction fragment length polymorphism analysis, allele-specific PCR, various variants of real-time PCR, DNA-array hybridization, mass-spectrometry analysis and DNA sequencing techniques. However, only sequencing being the direct method for nucleotide sequence determination allows unequivocally interpretation of the obtained results – nucleotide sequence of polymorphism region and homozygous or heterozygous nucleotide polymorphism.

Selection of the method for genetic polymorphism detection depends on tasks assigned to the laboratory including number of tests to be performed as well as the nucleotide sequence of the analyzed genome region. Since sequencing of small DNA fragment is sufficient for SNP detection use of Sanger sequencing technique or high-throughput genetic analysis systems is not always reasonable.

Pyrosequencing is the most convenient method for large-scale screening for known polymorphisms associated with various monogenic inherited diseases in cattle. The method enables determination of short-fragment

nucleotide sequences in genetic polymorphism region and known point mutations detection (single-nucleotide substitutions, one- or three-nucleotide deletions/insertions) by comparing to reference DNA sequence. The FGBU “VGNI” developed pyrosequencing-based methods for identification of the most common mutations in Holstein, Brown Swiss, Aberdeen Angus and Simmental cattle.

## MATERIALS AND METHODS

Genetic materials (frozen semen, venous blood) derived from domestic and foreign Holstein, Brown Swiss, Aberdeen Angus and Simmental bulls were used for testing.

DNA was extracted from venous blood with adsorption method using DNA-sorb-C-M kit (FGBI “Central Research Institute of Epidemiology”, Rospotrebnadzor) according to the manufacturer instruction. DNA extraction from frozen semen was carried out with DNA-sorb-C-M kit modified by the FGBU “VGNI”.

Primers for PCR and sequencing were designed using PyroMark Assay Design Software. Tertsik DNA-amplifier (DNA Technology, Russia) was used for PCR. Pyrosequencing was carried out with PyroMark Q96 MD (Qiagen, Germany) system using PyroMark Gold Q96 Reagents (50×96) kit (Qiagen).

## RESULTS AND DISCUSSION

Pyrosequencing-based methods for identification of the mutations associated with the most common monogenic inherited diseases in cattle were developed (Table 1).

At the first stage, analysis of the target gene nucleotide sequences from the NCBI OMIA database [10, 11] and literature [4, 7, 8] was carried out. Genome fragments associated with the particular inherited disease were selected. Oligonucleotide primers suitable for relevant genome fragment amplification in the mutation region as well as

**Table 1**  
Characteristics of the mutations identified with DNA pyrosequencing technology

Breed	Abbreviation	Disease	Polymorphism	Gene	Localization
Holstein	CVM	complex vertebral malformation	538G>T	SLC35A3	chromosome 3
	BLAD	bovine leukocyte adhesion deficiency	383A>G	ITGB2	chromosome 1
	DUMPS	deficiency of uridine-monophosphate synthetase	1213C>T	UMPS	chromosome 1
	CIT	citrullinemia	256C>T	ASS	chromosome 11
Brown Swiss	SMA	spinal muscular atrophy	562G>A	KDSR	chromosome 24
	SDM	spinal cord demyelination	560G>A	SPAST	chromosome 11
	W	Weaver syndrome	1703G>A	PNPLA8	chromosome 4
	BH2	Brown Swiss haplotype 2	757T>C	TUBD1	chromosome 19
Aberdeen-Angus	DD	developmental duplications	932T>C	NHLRC2	chromosome 26
	MA	alpha-mannosidosis	961T>C	MAN2B1	chromosome 7
	DW	dwarfism	2032C>T	PRKG2	chromosome 6
Simmental	BMS	bovine male subfertility	483C>A	TMEM95	chromosome 19
	TP	trombocytopeny	701A>G	RASGRP2	chromosome 29
	ZDL	zinc deficiency-like syndrome	702G>A	PLD4	chromosome 21
	AS	arachnomelia syndrome	1224_1225delCA (deletion)	MOC51	chromosome 23

primers for sequencing were selected using PyroMark Assay Design Software.

Forward and reverse sequencing can be performed depending on the nucleotide sequence of the analyzed fragment. Forward sequencing requires amplification reverse primer to be biotin-labelled at 5'-end, reverse sequencing requires biotin-labelled forward primers (Table 2).

Preparation of PCR-product for pyrosequencing includes incubation of amplicon with streptavidin-coated sepharose beads, alkaline denaturation for single-strand DNA generation, several consecutive washings using vacuum-filtering systems as well as sequencing primer annealing in the analyzed genetic locus region.

PCR-product sequencing is carried out with PyroMark Q96 MD system for genetic analysis. A set of enzymes (DNA-polymerase, luciferase, ATF-sulfurylase, apyrase), substrates (Luciferin, adenosine-5-phosphosulfate) and deoxynucleotides are used for pyrosequencing.

When matrix-complementary deoxynucleotide is gradually added to the reaction mix, DNA polymerase catalyzes the nucleotide incorporation into growing DNA chain that is accompanied with the release of pyrophosphate in a quantity equimolar to the amount of incorporated nucleotides. ATP-sulfurylase transforms pyrophosphate in ATF in the presence of adenosine-5-phosphosulfate. Resulting ATP triggers luciferin oxidation to oxyluciferin generating visible light emission proportional to the ATP amount. Chemiluminescent signal is detected and visible as peaks in pyrogram. The height of each peak is proportional to number of nucleotides in the matrix. Nucleotides are gradually added to the reaction mix according to the specified nucleotide sequence [5]. Analysis of obtained data are carried out at final stage of pyrosequencing.

PyroMark Q96 MD genetic analyzer software can be used for automated processing of the results since characterized polymorphic loci in cattle genome are examined and position of SNP is known. Figure 1 shows reference pyrograms for each of examined polymorphism variants in the TMEM95 gene. Mutation in the said gene is associated with bovine male subfertility (BMS) in Simmental bulls.

Figure 2 shows an example of pyrosequencing result interpretation. Polymorphism region is analyzed automatically using software based on relative signal heights within polymorphism region (compared to the signals corresponding to reference nucleotides). SNP-RUN programme is used to determine peak readings accuracy.

Only pyrograms of appropriate quality are used for the polymorphism allele frequency estimation. The following criteria for pyrogram quality evaluation were established:

1) analyzed sequence shall comprise specified number of nonvariable reference nucleotides. Analyzed sequence is a short fragment of DNA sequence comprising one or several polymorphisms to be analyzed. During analysis, reference peaks are used for assessment of peak heights within the polymorphism region and as an internal control for quality assessment. For example, in Figure 2 reference nucleotides are nucleotides at positions 2–4, 8–10;

2) absence of background signal at empty positions in the pyrogram automatically generated by the software programme. Empty positions indicate non-specific nucleotide insertion. Figure 2 shows that there are no nucleotides at positions 1 and 7 in the target nucleotide sequence, the nucleotides do not insert in generated chain and consequently signal level is equal to zero;

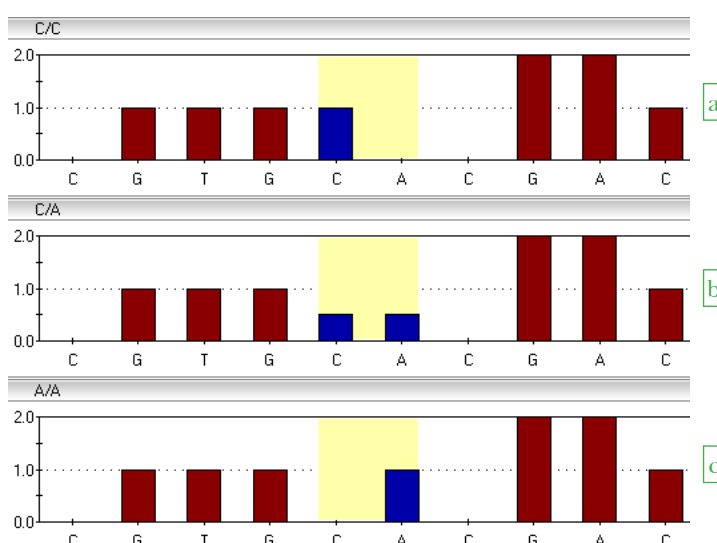
**Table 2**  
Methods for amplification of polymorphic genetic locus-containing fragments

Disease	Amplicon size, bp.	Type of analysis	Biotinylated primer
CVM	90	reverse	forward
BLAD	122	forward	reverse
DUMPS	115	reverse	forward
CIT	146	forward	reverse
SMA	140	forward	reverse
SDM	97	reverse	forward
W	96	reverse	forward
BH2	86	forward	reverse
DD	84	forward	reverse
MA	103	forward	reverse
DW	210	forward	reverse
BMS	136	forward	reverse
TP	170	reverse	forward
ZDL	183	reverse	forward
AS	105	reverse	forward

3) absence of background signal at variable positions (mutation region is indicated by colour in Figure 2);

4) sufficient signal intensity of target and reference peaks, a peak height shall be at least 30 relative light units (RLU) (value used in pyrosequencing for determination of peak heights in pyrogram). The peak height of 281 RLU is indicated in Figure 2a.

Genotype is identified according to the analyzed genetic locus based on relative height of target peaks at variable



**Fig. 1.** Reference pyrograms for identification of BMS-associated mutation (forward analysis)

a – normal genotype;  
b – heterozygote (carrier of the mutation);  
c – homozygous for mutant allele (mutant genotype).

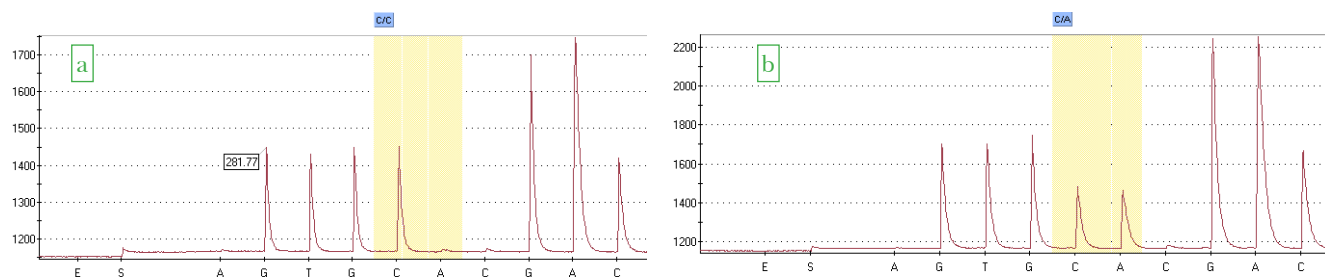


Fig. 2. Pyrograms generated by BMS mutation identification analysis (forward analysis)

a – normal genotype;  
b – heterozygote (carrier of the mutation).

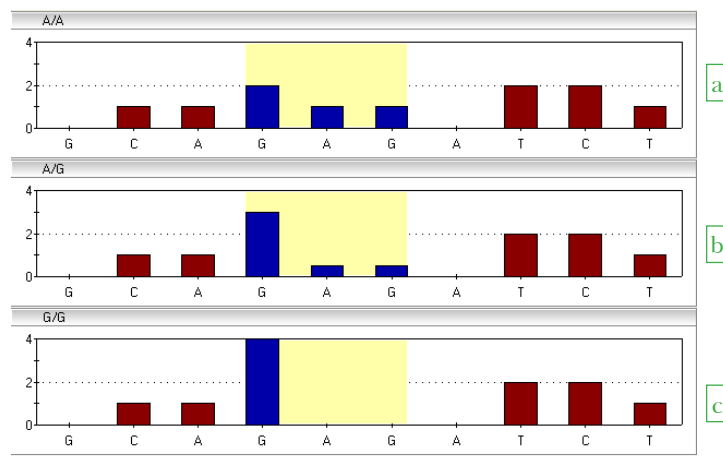


Fig. 3. Reference pyrograms for TP-associated mutation identification (forward analysis)

a – normal genotype;  
b – heterozygote (carrier of the mutation);  
c – homozygote for mutant allele.



Fig. 4. Reference pyrograms for TP-associated mutation identification (reverse analysis)

a – normal genotype;  
b – heterozygote (carrier of the mutation);  
c – homozygote for mutant allele.

position and conclusion on the animal genotype is issued. Identified genotype variants are as follows: homozygote for frequent allele (normal genotype), heterozygote, homozygote for rare allele (mutant genotype).

For example, results of analysis for BMS-associated mutation identification are interpreted as follows (Fig. 1):

- C/C – normal genotype (homozygous for normal allele, no mutation is identified);
- C/A – the animal is a carrier of the bovine male subfertility (BMS)-associated mutation in the TMEM95 gene (heterozygous, with one mutant copy and one normal copy of the gene);
- A/A – mutant genotype (homozygous for mutant allele).

Mutations associated with the following syndromes were detected in bulls tested with pyrosequencing-based techniques intended for single-nucleotide polymorphism monitoring: in Holstein cattle – 2% of the bulls were carriers of the CVM-associated mutation and 0.5% of the bulls were BLAD-mutation carriers; in Simmental cattle – 2% of the bulls were AS-mutation carriers, 5% of the bulls were BMS-mutation carriers and 7% of the bulls were TP-mutation carriers; in Aberdeen Angus cattle – 7.5% of the bulls were DD-mutation carriers; in Brown Swiss cattle – 5% of the bulls were SMA-mutation carriers.

Thus, pyrosequencing has several advantages. It allows rapid detection of many short DNA sequences, single nucleotide mutations, re-sequencing, etc. PyroMark Q96 MD genetic analysis system allows short DNA sequence identification, up to 96 samples within a short period. Simple chemical reactions and robust detection system make use of gels, electrophoresis and specific fluorescent labels unnecessary for the said method that significantly simplifies sample preparation procedure. Therewith, the analysis time and cost are significantly reduced as compared to capillary electrophoresis technique [2].

Besides, analysis of pyrosequencing data is simple and very clear. It includes comparison of output graph to the reference pyrogram proposed by the software.

The above-mentioned characteristics made the pyrosequencing technique the most suitable for rapid diagnosis of single nucleotide mutation in cattle genome located in the regions with known nucleotide sequence.

However, the technique has some disadvantages. Taking into account that DNA fragments intended for pyrosequencing should be no longer than 200 bp, the said technique is not suitable for diagnosis of the mutations associated with extended deletions or insertions. Therefore, a method based on PCR with electrophoretic detection was developed for identification of brachyspina syndrome, osteopetrosis and arthrogryposis multiplex in Holstein and Aberdeen Angus cattle. The said diseases are associated with extended deletions (more than 2,000 bp).

Pyrosequencing does not allow precise determination of identical nucleotides in DNA sequence when there are

more than 6 such consecutive nucleotides in the sequence. For example, mutation associated with arachnomyelitis and arthrogryposis in Brown Swiss cattle (SAA) is an insertion of one nucleotide, guanine (c.363–364insG), at the specified position in the SOUX gene after the 7 guanine nucleotide sequence [GGGGGGG]. Therefore, Sanger sequencing of the mutation region is required for identification of such SNP.

It should be noted that use of pyrosequencing technique for point mutation identification depends on the nucleotide sequence composition in the polymorphism region. In some cases, genotype identification based on tested genetic locus (normal genotype or carrier) is impossible. Thus, for example, reference pyrograms for thrombocytopeny identification in Simmental cattle are shown in Figure 3. The disease develops when adenine is substituted to guanine at position 701 of RASGRP2 gene in 29<sup>th</sup> chromosome. Correct identification of polymorphism variants based on relative height of target peaks at the substitution position with forward analysis is highly problematic (Fig. 3a and 3b).

In such case, the sequencing direction was changed to simplify the result interpretation. Reverse analysis makes polymorphism variant identification easy (Fig. 4).

## CONCLUSION

Control of genetic defects in cattle has become very important aspect of prevention in national veterinary practice and corrective selection in view of livestock breeding globalization and commercialization. Mandatory tests of breeding bulls for the breed-specific monogenic inherited diseases followed by recording of the mutation carriers in breed catalogues could further improve genetic potential of animals in the country.

Pyrosequencing technique allows mass screening for known polymorphisms associated with various monogenic inherited diseases in cattle. It enables identification of short-fragment nucleotide sequences in genetic polymorphism region and detection of known point mutations. Putting pyrosequencing-based genetic tests proposed by the FGBU "VGNKI" for identification of mutations associated with the most common inherited diseases in Holstein, Brown Swiss, Aberdeen Angus and Simmental cattle into practice as well as enhancing of farmers' awareness of the

problem and of the possibilities of its solving could minimize inherited disease spread and improve national cattle gene pool.

**Conflict of interests.** Authors declare no conflict of interest.

## REFERENCES

1. Introduction to molecular diagnostics: a study guide: in 2 volumes. Vol. 2: Molecular genetics methods in the diagnosis of inherited and oncology diseases [Vvedenie v molekulyarnuyu diagnostiku: uchebno-metodicheskoe posobie: v 2 t. T. 2: Molekulyarno-geneticheskie metody v diagnostike nasledstvennyh i onkologicheskikh zabolevanij]. Ed. by M. A. Paltsev, D. V. Zaletaev. M.: Meditsina, 2011 (in Russian).
2. Genetic polymorphisms and risk of developing certain multi-factor diseases [Geneticheskie polimorfizmy i risk razvitiya nekotoryh mul'tifaktornyh zabolevanij]. E. S. Potekhina, K. O. Mironov, O. P. Dribnokhodova [et al.]. *Spravochnik zaveduyushchego KDL*. 2014; 10: 37–47 (in Russian).
3. Zhigachev A. I., Ernst L. K., Kudryavtsev V. A. Genetic diseases in Black-and-White, Holstein and Ayrshire cattle and prevention thereof [Geneticheskie bolezni u cherno-pestrogo, golshtinskogo i ajshirskogo skota i ih profilaktika]. *Issues of Legal Regulation in Veterinary Medicine*. 2008; 1: 46–56 (in Russian).
4. Monogenic hereditary defects and their role in reproduction [Monogennye nasledstvennyye defekty i ih rol' v vosproizvodstve]. N. Zinovyeva, N. Strekozov, G. Eskin [et al.]. *Zhivotnovodstvo Rossii*. 2015; 6: 30–31 (in Russian).
5. Experience of using pyrosequencing-based genetic analysis systems [Opyt ispol'zovaniya sistem geneticheskogo analiza na osnove tekhnologii pirosekvenirovaniya]. K. O. Mironov, E. A. Dunaeva, O. P. Dribnokhodova, G. A. Shipulin. *Spravochnik zaveduyushchego KDL*. 2016; 5: 33–43 (in Russian).
6. Main genetic causes of embryonic loss in dairy cattle husbandry associated with intensive selection based on performance [Osnovnye geneticheskie prichiny embrional'nyh poter' v molochnom skotovodstve, svyazannye s intensivnoy selekciej po produktivnosti]. S. V. Guskova, I. S. Turbina, G. V. Eskin, N. A. Kombarova. *Dairy and Beef Cattle Farming*. 2014; 3: 10–14 (in Russian).
7. Cole J. B., Null D. J., VanRaden P. M. Phenotypic and genetic effects of recessive haplotypes on yield, longevity, and fertility. *J. Dairy Sci.* 2016; 99 (9): 7274–7288; DOI: 10.3168/jds.2015-10777.
8. Genetic disorders in beef cattle: a review. A. Ciepłoch, K. Rutkowska, J. Oprządek, E. Poławska. *Genes Genom.* 2017; 39 (5): 461–471; DOI: 10.1007/s13258-017-0525-8.
9. Mendelian Inheritance in Cattle 2000. ed. P. Millar, J. J. Lauvergne, C. Dolling Waseningen: EAAP publication, 2000: 101.
10. National Center for Biotechnology Information (NCBI). URL: [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov) (date of access: 13.09.19).
11. Online Mendelian Inheritance in Animals (OMIA). URL: [www.omia.org/home](http://www.omia.org/home) (date of access: 13.09.19).

Submitted on 26.09.19

Accepted for publication on 25.12.19