PhD THESIS

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The polymorphism in GDF8 gene and its association with meat performance in the chosen sheep breeds

Polimorfizm genu GDF8 i jego powiązanie z cechami użytkowości mięsnej wybranych ras owiec

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Wszystkim osobom życzliwym
składam serdeczne podziękowania
za okazaną pomoc i wsparcie
w trakcie pisania niniejszej pracy

Moim Najbliższym
serdecznie dziękuję
za wsparcie, pomoc i zrozumienie
okazane w trakcie pisania pracy

CONTENTS

1. INTRODUCTION AND THE AIM OF THE THESIS

Sheep have been used by humankind in order to obtain meat, milk and wool. In Poland at first the main propose of rearing those animals was to obtain wool, therefore, the decrease in the price of wool after the 1990s resulted in the decrease of the number of sheep. Nowadays, the most profitable direction in sheep husbandry is meat production (Rokicki, 2008). Despite many health promoting properties of lamb meat (Milewski, 2006), there is no demand for this kind of meat on Polish national market. After accession to the European Union, Poland however gained the access to a market that may accept Polish lamb meat. And so, nowadays Poland is mainly dependant on the opportunity to export the meat to the Untied European market (Rokicki, 2008), therefore it is important for Polish meat to be competitive in terms of price and quality.

The performance traits of animals are very important as they affect profitability of production. For centuries the only way of improving the meat performance was to select individuals with the favourable phenotypic features and using them in breeding. Nowadays however, the process may be supported by the use of modern techniques of molecular genetics. Identification of quantitative trait loci (QTL) for meat performance in different species of animals has been an important part of the research for many years. In order to improve breeding programmes, researchers have tried to identify genes that are associated with production traits and evaluate the impact of different polymorphisms in those genes. Different techniques that facilitates detection of various mutations are available, one of them being PCR-RFLP, PCR-SSCP and sequencing. All three techniques were used during the present study.

Sheep breeding programmes in Poland focus mainly on meat traits. Therefore, it is important to understand the genetic basis for such performance. It has been found that animals meat performance is affected by many genes, one of which is the myostatin gene, that has been associated with the double muscle (DM) phenotype in cattle i.e. Belgian Blue or Piemontese breed (Wiener and Gutierrez-Gil, 2009; Allais *et al*., 2010). It has also been associated with hypertrophy in sheep. This gene is called Growth Differentiation Factor 8 (*GDF*8) or *MSTN*, and is located on ovine chromosome 2. It encodes the myostatin protein, which acts as the negative regulator of skeletal muscle growth. Any polymorphism in this gene, that disrupts expression of the gene, changes protein conformation or its concentration in blood may cause an increase in muscle weight (McPherron *et al*., 1997; Pas Te *et al*., 2004). Nowadays, several mutations that cause changes in meat performance are known, however, as literature suggests, polymorphism in myostatin gene and its association with meat performance may be breed-dependant (Lee and McPherron, 2001; Kijas *et al*., 2007; Hadjipavlou *et al*., 2008; Bignell

et al., 2009; Boman *et al*., 2009; Johnson *et al*., 2009). Therefore, it is important to identify myostatin genotypes of sheep breeds reared in Poland and change breeding programmes accordingly in order to improve meat performance.

The aim of the thesis was to identify polymorphisms in two fragments of ovine myostatin gene and to evaluate the frequencies of myostatin alleles and genotypes in three Polish sheep breeds: Kamieniec, Pomeranian and Coloured Polish Merino. Another aim of the study was to investigate the association between polymorphisms in *GDF*8 gene and meat performance traits, some of them being body weights at different days of lamb's life, average daily gains between the 2^{nd} and 56^{th} day of lamb's life and carcass traits.

2. OVERVIEW OF THE LITERATURE

2.1. SHEEP HUSBANDRY IN POLAND

Sheep (*Ovis aries*), ruminant mammals that are widely used as livestock, belong to an order of even-toed ungulates (*Artiodactyla*), bovid family **(***Bovidae*) and the genus *Ovis*. For centuries farmers have been rearing those animals mainly for their meat, milk and wool, but also in order to obtain fertilizers. In the south of Poland for generations sheep's milk has been used to produce oscypek (a smoked cheese characteristic for the Tatra mountain), bryndza (a sheep milk cheese) and their fleece has been used for coats production (Krupiński, 2008; Szymańska, 2009). Moreover, a sheep meat is a good source of high quality protein, minerals (e.g. iron, zinc, phosphorus, calcium) and vitamins (B_1, B_2, B_6, B_{12}, PP) (Milewski, 2006). It contains omega-3 fats and conjugated linoleic acid (CLA) that contribute to human's health, for instance, by inhibiting neoplasia and atherosclerosis, acting as antioxidants, stimulating the immune system, preventing obesity and by lowering body fat level. Lamb meat is easily digestible (Milewski, 2006).

Throughout the centuries, the number of sheep reared in Poland has change greatly. Those changes were mainly caused by political and economic reasons. The first records of sheep reared on Polish lands date back to the XIII century, and suggest that back then farmers kept long-wool sheep breeds. In Red Ruthenia, now a part of Western Ukraine that in medieval times used to belong to Poland, sheep breeds such as Wrzosówka and Krukówka were reared. Moreover, farmers started to develop new breeds that were characteristic for the particular regions, for instance, Pomeranian sheep appeared in the region of Pomerania, Wielkopolska sheep in the region of Wielkopolska and in mountains – Mountain sheep. Historical records indicate that during the XIX century sheep farming in Poland bloomed as a result of the increased demand for sheep wool in Europe (Szymańska, 2009). However, after 1986 the population of sheep in Poland decreased due to the economic changes in the Central and Eastern Europe which resulted *inter alia* in decrease in the profitability of sheep production and reduction of the demand for wool. Polish wool could not stand the competition with wool imported from Australia or New Zealand, which was cheaper and of better quality. All this resulted in the dramatic decrease in the number of sheep. Farmers acknowledged the fact that the they had to change the focus of the production, and therefore, some programmes that aimed to convert the domestic production into meat-focused one were designed. In 1996 the "The Programme of Sheep Improvement by the Year 2010" („Program rozwoju krajowego owczarstwa do roku 2010") was proclaimed. The aim of the programme was to reconstruct the sheep population in Poland. However, the aim was not achieved, which might have been caused

by the decreases in funding sheep flocks by the Fund for Biological Progress (Fundusz Postępu Biologicznego) (Rokicki, 2008). Nowadays, the sheep husbandry in Poland is focused mainly on meat production. But since there is no demand for the lamb meat in the domestic market, most of the production goes abroad. The size of production is therefore largely dependent on opportunity to export the meat to the Western countries (e.g. to Italy, Netherlands, Germany) (Dankowski *et al.*, 2005; Rokicki, 2008).

Total sheep population in Poland recorded in July 2015 reached the level of 227.7 thousand individuals, which was higher by 2.2% (4.9 thousand individuals) than the level recorded a year before and by 13.1% compared to the number of sheep recorded in December 2014. The number of ewes increased by 10.2 % (13.7 thousand individuals) (Table 1) (GUS, 2015). According to the Main Statistical Office (Główny Urząd Statystyczny, GUS,) in June 2014, 222.8 thousand sheep were reared in Poland, which was lower than the number from previous June by 26.7 thousand individuals (10.7%) (GUS, 2014). The Graph 1 presents the change in the number of sheep in Poland between June 2003 and June 2015.

Among all the breeds reared in Poland for the purpose of the present study three Polish sheep breeds i.e. Kamieniec, Pomeranian and Coloured Polish Merino have been chosen. Table 2 presents the number of sheep of those three breeds subjected to the Genetic Resources Conservation Programmes in Poland (2008-2013).

2.1.1. Kamieniec sheep

One of the sheep breeds reared in Poland is a long-wool sheep called Kamieniec (Kawęcka and Sikora, 2012). The work on that breed was initiated after the first World War by professor Stanisław Jełowicki. Kamieniec breed was created by crossbreeding the primal Pomeranian ewes with the Texel or Leine rams and subsequently with Kent sheep. In 1972 professor Jełowicki provided the description of the breed characteristics and its name (Milewski, 2014).

Kamieniec sheep is one of the most prominent conservative races in Poland (Milewski, 2014). Currently, the selection of Kamieniec sheep is focused mainly on the meat and wool performance (Kawęcka and Sikora, 2012). Those animals are characterised by a good milk production and prolificacy as well as a good meat performance. The quality of lamb meat is considered to be very good. Kamieniec sheep have large and broad bodies and they are well fleshed. Their wool is white and it covers most of their bodies. Fleece is uniform in quality (Laudowicz, 1977; Lipecka and Gruszecki, 2006a). Kamieniec sheep are well adapted to the severe climatic conditions that are natural for the north-eastern part of Poland, therefore, they may be reared on the natural pastures

located in vicinity of lakes and rivers (Kawęcka and Sikora, 2012; Milewski, 2014). Table 3 provides information about the characteristics of performance traits of Kamieniec Sheep.

The breed has been included into the Genetic Resources Conservation Programmes for sheep due to several reasons. First of all, the animals are the part of the Warmia and Masuria landscape, and they help to maintain the biodiversity in other parts of the country. They may be used in agriculture farms and their wool and skin may be used by local craftsmen (Milewski, 2014).

In 2013 the number of Kamieniec sheep in Poland subjected to the Genetic Resources Conservation Programme reached the level of 3,388 heads (Instytut Zootechniki, 2013). Most of them are kept in Warmia-Masuria, Masovian, Podlaskie and Pomeranian Voivodaships.

2.1.2. Pomeranian sheep

Pomeranian sheep is a native Polish long-wool sheep, which is subjected to the Genetic Resource Protection Programme. The breed was developed on the basis of primary primitive Pomeranian sheep that were characterised by the body weight of ewes on the level of 50 kg and had thick wool (Szczepański *et al.*, 2001). Those sheep were crossbred with Texel, Leine and Kent sheep. Between 1925 and 1928 Friesian breeds and later in 1929-1935 Holstein breeds were used to improve the native breed. After the second World War only Texel sheep were used for ameliorating Pomeranian sheep. The work resulted in improving wool quality, meat performance, prolificacy and caused accelerated sheep maturation (Laudowicz, 1977; Baranowski, 1998).

Pomeranian sheep are resistant to diseases, have low nutritional requirements and produce tasty meat of a high quality. Moreover, ewes are characterized by a high milk yield (Lipecka and Gruszecki, 2006b; Siminska *et al.*, 2008; Kawęcka and Sikora, 2012). Pomeranian sheep are proportionally built, they are rather large animals with long and wide bodies and well-developed legs. Their fleece is half open, white and it covers their whole body except for the head and legs. The meat obtained from this breed is considered to be tasty (Laudowicz, 1977). In 2011 the number of Pomeranian sheep in Poland was around 8,500 (Kawęcka and Sikora, 2012), while 6,511 individuals were subjected to the Genetic Resources Conservation Programme (Instytut Zootechniki, 2013). Table 3 provides information about the characteristics of performance traits of Pomeranian Sheep.

2.1.3. Coloured Polish Merino sheep

Colour variety of Polish Merino Sheep (Coloured Polish Merino) was established in the 1980s in the Zootechnical Experimental Station in Kołuda Wielka (the Kuyavian-Pomeranian Voivodeship, Poland). The work was supervised by professor M. Osikowski (Lipecka and Gruszecki, 2006c). At the beginning sheep with coloured fleece (animals with the genotype BB) were selected from the herd of Merino sheep and crossbred with white individuals (NN) which resulted in birth of white lambs that carried coloured trait allele (genotype BN). Those animals were further crossbred with white Merino sheep, and 50% of lambs were homozygous for the coloured fleece trait allele (BB). The third stage of establishing a breed was to crossbred coloured animals (BB) which resulted in obtaining animals that were homozygous for the trait (1,005 of BB individuals). In addition to improving the meat performance of Merino sheep, the purpose of establishing the new breed was to provide naturally coloured wool and sheepskins for ecological coats and accessories. Another aim was to investigate the inheritance principles of coloured fleece (Pakulski, 2010; Kawęcka and Sikora, 2012).

Individuals of Coloured Polish Merino are usually black, sporadically grey. Almost all animals have a white spot on the top of the head (Pakulski, 2010; Kawęcka and Sikora, 2012). Coloured Polish Merino sheep are characterised by a good meat performance. The table 3 presents information about the characteristics of performance traits of that breed. In 2013 there were 344 Coloured Polish Merinos subjected to the Genetic Resources Conservation Programme (Instytut Zootechniki, 2013).

2.2. MYOSTATIN GENE, ITS POLYMORPHISM AND ASSOCIATION WITH MEAT PERFORMANCE

The fragment of the DNA that affects a complex quantitive trait is called the quantitive trait loci (QTL). A quantity trait is a phenotypic trait that can be affected by a gene, polygene (two or more genes) as well as by the environment (Nicholas, 2010; Hartl and Ruvolo, 2012). Identification of genes that influence phenotypic traits may be achieved by investigating candidate genes that are already known to be associated with the physiological or development processes that effect the trait. Once the particular region is identified as the one affecting the trait, it may be investigated even further. Researchers may identify the genetic basis of favourable phenotype, design markers that facilitate identification of the desirable genotype, which later may be used by farmers to enhance the breeding progress (Hartl and Ruvolo, 2012).

Identification of quantitative trait loci (QTL) for meat performance in different species of animals has been an important part of the research for many years. Performance traits are economically important as they affect profitability of the production. In order to improve breeding programmes, researchers have tried to identify genes that are associated with production traits and evaluate the impact of different polymorphisms in those genes (Słomski *et al.*, 2008). Nowadays in Poland the sheep husbandry focuses mainly on meat production, therefore, a good meat performance is crucial for profitability. It has been found that animals meat performance is affected by many genes. One of which is myostatin that has been associated with the double muscle (DM) phenotype in cattle i.e. Belgian Blue or Piemontese breed. It has also been associated with hypertrophy in sheep (Lee, 2004; Boman *et al.*, 2009).

A QTL for a muscular hypertrophy in sheep has been located on the ovine chromosome 2 in the region that contains the myostatin gene (Boman *et al.*, 2009). The myostatin gene (*GDF*8, also called *MSTN*) encodes a protein that belongs to the superfamily of the transforming growth factor β (TGFβ) and is synthesized primarily in skeletal muscles. The protein acts as a negative regulator of the skeletal muscle growth (Lee, 2004).

TGFβ superfamily is a large group of structurally related regulatory proteins that can be divided into two groups: TGF-β/Activin and BMP/GDF (which stands for bone morphogenetic protein/growth and differentiation factor). Those groups may be further divided according to similarity in their sequence and evolutionary conserved molecules. Proteins from that family are synthesized as a precursor molecules and the mature protein consists of disulfide-linked dimmers. Myostatin protein belongs to the *GDF*8 subgroup (Caestecker, 2004).

2.2.1. The discovery of the myostatin gene

For the first time *GDF*8 was described in mice by McPherron *et al.* in 1997. They conducted a study in which inhibited expression of this gene resulted in an increase in the number and the size of muscle fibers (McPherron *et al.*, 1997). Mice with the knockout *GDF*8 gene were found to be 25-30% heavier than individuals of the wild type, whilst their individual muscles weighted even two times more than those of mice that carried *GDF*8 gene (Lee and McPherron, 1999). Elkasrawy and Hamrick (2010) used mice to build the model of the interaction between muscle mass and bone strength. They found that the animals without *GDF*8 gene, in addition to the increased muscle weight and reduced fat content, were characterized by a greater bone strength. Moreover, some studies were conducted in order to diagnose the mutation responsible for the DM phenomenon in cattle, *inter alia*, in Belgian Blue breed (Dunner *et al.*, 2003). Since the moment of the discover,

the myostatin gene was described by many authors and the association between the gene and animals' performance was investigated extensively in many species and breeds (Dunner *et al.,* 2003; Schuelke *et al.,* 2004; Clop *et al.,* 2006; Boman *et al,.* 2009; Hickford *et al.,* 2009; Kemaladewi *et al.,* 2011).

2.2.2. Myostatin gene's structure and polymorphisms

The gene encoding myostatin protein is called Growth Differentiation Factor 8 (*GDF*8 or *MSTN*). It has been located on ovine chromosome 2 (Chromosome 2: 118144443-118149433 forward strand). It is composed of 3 exons and 2 introns. First exon is composed of 373 bp, second of 374 bp, third of 381 bp, whilst introns one and two consist of 1,833 and 2,030 bp, respectively (Ensemble, 2016). The structure of the myostatin gene is presented in the Figure 1. *GDF*8 encodes the myostatin protein, which consists of 375 amino acids (Boman *et al.,* 2009).

Myostatin protein, encoded by the myostatin gene (*GDF*8), is synthesized primarily in skeletal muscles as a precursor protein with a mass of 42,827 Da (Kambadur *et al.,* 1997; Boman *et al.,* 2009; Uniprot 2015). The precursor protein consists of 375 amino acids (Kambadur *et al.,* 1997; Boman *et al.,* 2009), out of which first 23 create a signal peptide, further 243 create propeptide (Latency Associated Peptide, LAP) and only remaining 109 amino acids create actual myostatin chain (mature 12 kD apolipeptyde) (Tellam *et al.,* 2012, Uniprot, 2015). LAP is non-covalently linked to the mature protein and it prevents the protein from binding to its receptor (Caestecker, 2004). A mature myostatin protein creates a dimer and both a dimer and a propeptide circulate in the bloodstream. Figure 2 presents the structure of myostatin protein.

According to Li *et al.* (2010) enhancement of muscle mass in mice may be induced by over-expression of LAP since LAP may bind myostatin and inhibit myostatin signalling activity (Tellam *et al.,* 2012). Myostatin C-terminal dimer may bind to the activin type II receptors (ActRIIB) and to some extent to ActRIIA. This may be inhibited by follistatin (a activin-binding protein) and by myostatin propeptide (Lee and Mcpheron, 2001; Kemaladewi *et al.,* 2011) Binding myostatin to ActRIIB may result in activating SMAD signalling transduction pathway (Elkina *et al.,* 2011). SMAD belongs to intracellular protein family that transduce extracellular signals from TGFβ ligands to the nucleus. They activate gene transcription. Activating the SMAD pathway results in altering transcription of the *GDF*8 and inhibiting muscle growth (Elkina *et al.,* 2011). In addition, myostatin activates some cyclin-dependent kinase pathways, which negatively affects prenatal and postnatal myogenesis i.e. the cell cycle of myoblasts and myoblast differentiation (Langley *et al.,* 2002; McCroskery *et al.,* 2003; Pas Te *et al.,* 2004)

The polymorphism in the myostatin gene has been described widely in the literature due to its association with the meat performance. The polymorphic variants are described extensively especially in cattle (Wiener and Gutierrez-Gil, 2009; Allais *et al.,* 2010). The best known example of double muscled animals are cows of the Belgian Blue breed. The breed is characterized by the increased of the number of the muscle fibers. These animals are therefore considerably larger than other cattle breeds. Scientists investigating this phenomenon discovered that the muscled phenotype of Belgian Blue is associated with the polymorphism in the myostatin gene. The nt821 (del11) mutation in the coding region of the *GDF*8 gene causes the deletion of 11 nucleotides in exon 3 which results in reading frame shifting (frameshift mutation) and creating premature STOP codon. Since the resulting protein is shorter than the normal one it is biologically inactive and animals with such mutation exhibit more muscled phenotype. The nt821 (del11) mutation has been described in several cattle breeds, *inter alia*, in Blonde d'Aquitaine, Limousine, Parthenaise, Rubia Gallega, Red Angus (Karim *et al.,* 2000; Wiener and Gutierrez-Gil, 2009; Allais *et al.,* 2010). Another mutation described in the literature is nt419 (del7-ins10), which stands for the deletion of 7 nt with the insertion of 10 nucleotides in the second exon of the *GDF*8 gene. It results in creating the premature STOP codon. The protein is inactive as its translation stops at the 140th amino acid. The mutation has been described in Mmaine-Anjou cattle breed (Grobet *et al.,* 1998). In breeds such as Piedmontese, and Gasconne scientists described C313Y mutation. The G>A transition at the 938th nucleotide (exon 3 of the *GDF8* gene) results in changing the three-dimensional protein structure which causes the loss of biological activity (Kambadur *et al.,* 1997; Grobet *et al.,* 1998; Karim *et al.,* 2000; Bellinge *et al.,* 2005). The Q204X mutation, described in Charolaise cattle, is a C>T transition that by creating premature STOP codon results in the creation of a shorter protein (204 amino acids) which lacks biological activity. Animals with that mutation are better muscled (Karim *et al.,* 2000; Allais *et al.,* 2010). Another mutation that has been found to have an influence on cattle meat performance is the E226X mutation. This mutation has been describes so far in Maine-Anjou cattle. It is characterized by the G>T transition at the 676th nucleotide. Animals with the E226X mutation produce inactive protein (Grobet *et al.,* 1998; Karim *et al.,* 2000).

The polymorphisms in the *GDF*8 gene and their association with the meat performance have been acknowledged also in sheep husbandry. Scientists aim to describe mutations in the myostatin gene that affect meat performance. One of the best known mutation occurring in sheep is c.*1232G>A mutation. The decrease in the level of myostatin circulating in blood to even 1/3 of a normal level has been associated with the G>A transition in 3'UTR of *GDF*8 gene (Clop *et al.,* 2006; Boman *et al.,* 2010; Georges, 2010).

The mutation creates the microRNA illegitimate target site, which results in inhibiting myostatin translation (Clop *et al.,* 2006). The A allele (also called c.*1232A) has been associated with enhanced meat performance. Animals with the genotype AA are more muscled that heterozygotes, which are better in terms of meat performance than animals with GG genotype (Han *et al.,* 2010). The mutation has been investigated in various sheep breeds, *inter alia*, in New Zealand (NZ) Texel, Australian Texel, Poll Dorset, East Friesian and Charollais (Kijas *et al.*, 2007; Hadjipavlou *et al.,* 2008; Bignell *et al.*, 2009; Johnson *et al.*, 2009). Moreover, in Norwegian White Sheep scientists identify the c.960delG mutation. It is characterized by a deletion of the guanine at the position 960. It is a frameshift mutation that results in creating premature STOP codon. Animals with that mutation are known to be more muscled (Boman *et al.*, 2009). Another mutation described in Norwegian Spælsau sheep is the insertion of adenine in the position of 120th nucleotide (c.120insA). The protein is shorter than normal one and lacks biological activity (Boman and Vage, 2009).

Moreover, Hickford *et al.* (2009) investigated SNPs in the intron 1 of the *GDF*8 gene. They used PCR-SSCP method to identify 5 SSCP alleles (A, B, C, D and E). The A allele has been associated with the decrease in the muscle weight, contrary to the B allele which has been associated with the improvement in meat performance (Hickford *et al.,* 2009). Also Ansary *et al.* (2011) used PCR-SSCP method to investigate polymorphism in *GDF8* gene. They described 3 SSCP patterns (P1, P2, P3) in Iranian Baluchi Steep. The animals with the P1 genotype where better in terms of body weight (Ansary *et al.,* 2011).

The literature provides information about many mutations in the *GDF*8 gene that are linked to meat performance. However, it also suggests that the mutation frequency and the impact of those changes in the DNA may be species and breed dependant. Therefore, each breed has to be investigated separately in order to determine the effect of the polymorphism.

2.3. EFFECT OF DIFFERENT FACTORS ON SHEEP MEAT PERFORMANCE TRAITS

Sheep meat performance is affected by many factors, one of them being genetics. Moreover, diversity in meat performance is caused also by environmental factors, food management and year of birth. Many authors indicate that the breed and even the herd membership have a huge impact on sheep's body weight (Piwczyński and Mroczkowski, 2005; Gardner *et al.*, 2007; Guerrero *et al.,* 2013). It is widely known that sex dimorphism is another factor that has to be taken into consideration during any study concerning meat performance. It has been noticed that males tend to be bigger at birth than females. Moreover, type of birth (litter size) is another agent that affects lamb's

body weight. Singletons have an advantage in terms of body weight over animals born in a litter as the body weight of an individual decreases with the increasing litter size (Piwczyński and Mroczkowski, 2005; Gardner *et al.*, 2007; Guerrero *et al.*, 2013).

2.4. METHODS USED IN ANALYSING POLYMORPHISM – GENERAL IDEAS

The production traits of animals are very important as they affect profitability of production. For centuries the only way of improving the meat performance was to select individuals with the favourable phenotypic features and using them in breeding. Nowadays however, the process may be supported by the use of modern techniques of molecular genetics. The identification of QTL and finding their association with the animals performance may help to accelerate breeding progress and increase profitability of the production. In sheep husbandry different molecular tools can be used, for example PCR-RFLP and PCR-SSCP techniques. They allow to identify alleles and genotypes of individuals and select only those with the favourable ones. One of the basic tools of molecular genetics since its introduction in 1980's is PCR, which stands for the Polymerase Chain Reaction (Słomski *et al.*, 2008). PCR is a method that allows to amplify the number of copies of the target DNA region (Awole Adem, 2006; Słomski *et al.*, 2008).

RFLP, which stands for Restriction Fragments Length Polymorphism, is a molecular technique widely used for detecting DNA polymorphisms. It allows to detect DNA mutations that eliminate or create new restriction enzyme sites. Such mutations change the number and lengths of digested PCR products (Kakavas *et al.*, 2008; Słomski *et al.*, 2008). In animal breading the PCR-RFLP reaction is often used for identifying genotypes that are associated with qualitative and quantitative traits. It is also used to determine whether the individual caries an allele responsible for resistance to certain pathogens or diseases (Słomski *et al.*, 2008). The advantage of the RFLP method is its simplicity. Moreover, it is a cheap technique that normally does not require the purchase of expensive equipment. In order to perform RFLP one needs only a standard equipment that is available in every laboratory, namely, thermocyclers used for PCR, equipment for electrophoresis and visualization of the results. The disadvantage of the technique is the fact that it cannot be used for identification of new polymorphisms. Moreover, it is not suitable for simultaneously testing multiple mutations that are located in different genes, since RFLP requires the usage of specific primers and restriction enzymes. Therefore, for testing different gene fragments it is necessary to use different primers and restriction enzymes (Rasmussen, 2012).

Another method that is being used in animal genetics is SSCP (Single Strand Conformation Polymorphism). Since its development in 1989, the technique is used for detecting already known DNA mutations or for screening DNA looking for new polymorphisms (Orita *et al.,* 1989; Kakavas *et al.,* 2008). The principle of this technique is based on the fact that in the nondenaturating (native) electrophoresis the single strand DNA fragments (ssDNA) undergo a 3-dimensional folding assuming conformational state. The conformation is based on DNA sequence. Different particles of DNA migrate in gel with different speed and therefore, create different band pattern. Every single change in the DNA sequence may lead to change of conformational state, which means that a new allele would have different 3-dimensional folding compared to the wild one and SSCP patterns for both would differ (Charon and Świtoński, 2006; Kakavas *et al.,* 2008; Słomski *et al.,* 2008). The main advantage of SSCP technique is the fact that silver nitrate is more sensitive than ethidium bromide, which allows to detect lower concentrations of DNA in the gel (Charon and Świtoński, 2006). Moreover, it is possible to extract DNA from the stained gel and use it for subsequent tests including sequencing. By using SSCP method it is possible to screen a large number of samples in a cost-efficient way in order to detect mutations or find new, unknown polymorphisms. The disadvantage of the technique is the inability to identify the type of mutation. In order to do so, it is necessary to use other molecular genetics techniques such as sequencing. However, when the type of mutation is known, the SSCP method allows to test a great number of samples in a relatively short time (Charon and Świtoński, 2006; Słomski *et al.,* 2008).

3. MATERIAL AND METHOD

3.1. ANIMAL MATERIAL

The study involved the population of 297 individuals belonging to three different sheep breeds: Kamieniec, Pomeranian and Coloured Polish Merino. Animals varied in terms of sex and type of birth. The number of sheep used in this study is presented in the table 4 along with the distribution of sex and type of birth in three tested sheep breeds.

Sheep of Pomeranian breed were kept in Pomeranian voivodeship (near Łeba). The flock of Kamieniec sheep was owned by the Production and Experimental Station in Bałcyny and was a leading conservation flock of Kamieniec sheep in the Warmińsko-Mazurskie voivodeship, and one of the best in Poland. The biological material from Kamieniec and Pomeranian sheep was collected and shared by employees of Department of Sheep and Goat Breeding, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn. The Coloured Polish Merino sheep were kept in the National Research Institute of Animal Production, Experimental Station in Kołuda Wielka.

3.2. BLOOD COLLECTION AND DNA ISOLATION

Blood samples were collected in 2012 by a veterinarian. The peripheral blood was collected from jugular vein, and stored in blood collection tubes with K^2EDTA (Profilab, Poland). Samples were stored in 4ºC.

The genomic DNA was extracted from peripheral sheep blood with the use of the MasterPure™ DNA Purification Kit for Blood (Epicentre Biotechnologies, USA) according to manufacturer's methodology with modifications suggested by Grochowska (2016) (250 µl of blood was taken and placed in sterile Eppendorf tubes (of 1.5 ml volume)). Samples were stored in 4ºC or -20 ºC.

3.3. QUANTITATIVE AND QUALITATIVE EVALUATION OF ISOLATED DNA

Quantitative and qualitative evaluation of isolated DNA was performed by using spectrophotometer NanoDrop 2000 (Thermo Scientific, USA). DNA concentration (µg/ml) and DNA purity (the ratio of the absorbance at 260 and 280 nm, A_{260}/A_{280}) were recorded. The data was used for preparing DNA dilutions, by adding appropriate amount of TE buffer in order to achieve the concentration of 50 ng/µl.

At the same time, all DNA samples were separated in 2% agarose gel (Prona Agarose, Spain) with the addition of Midori Green DNA Stain (Nippon Genetics Europe GmbH, Germany: 0,5 µl Midori Green was used per 10 ml 1XTBE buffer; 10xTBE: 0.89M Tris, 0.89M boric acid, 0.02M EDTA, pH 8.0). 5 µl of DNA solution mixed with 2 µl of 6XLoading Dye Solution (Fermentas UAB, Lithuania) was loaded on the gel. The electrophoresis was carried out in 1XTBE running buffer, for 60 minutes at 120V (electrophoresis equipment produced by Cleaver Scientific LTD, power adapter by Major Science). The DNA bands were visualized by using UV transilluminator (wavelengths of 312 nm) and archived by the G:Box Chemi XR5 (Syngene, United Kingdome).

3.4. MOLECULAR ANALYSIS

3.4.1. DNA fragments amplification

Amplifications of two DNA fragments were performed. For each individual, two separate PCR reactions named MioClop and Hickford PCR were made. Two different sets of primers were used:

- for MioClop PCR: MioClop_F and MioClop_R (Genomed S.A., Poland) (Clop *et al.*, 2006)
- for Hickford PCR: Hickford_F and Hickford_R (Genomed S.A., Poland) (Hickford *et al.*, 2009)

The data concerning primers characteristics and PCR products lengths is presented in table 5, while the composition of the reaction mixtures (Grochowska, 2016) is presented in table 6. PCR was carried out in Mastercycler pro S thermocycles (Eppendorf, Germany). PCR parameters according to Grochowska (2016) methodology are presented in table 7.

After amplifying gene fragments, the electrophoresis was performed for each sample in order to assess the quality of obtained PCR products. The separation was run in 2% agarose gel (Prona Agarose, Spain), with the addition of Midori Green DNA Stain (Nippon Genetics Europe GmbH) at 120V for 60 minutes (Cleaver Scientific LTD). The solution consisting of 3 µl of PCR products and 2 µl of 6XLoading Dye Solution (Fermentas UAB, Lithuania) was loaded into the gel. Two different markers were used depending on the type of the PCR fragments: pUC19 DNA/*Msp*I (*Hpa*II) Marker (Life Technologies, USA) (when Hickford PCR was carried out) and Lambda DNA/*Hind*III Plus Marker (Life Technologies, USA) (when MioClop PCR was performed). Gels were analysed and results were archived by G:Box Chemi XR5 (Syngene, United Kingdome). The PCR product resulted from amplification with the use of MioClop primers had a length of 1,003 bp, while the second product, obtained after the usage of Hickford primers, was 414 bp

long. One single band attested to the accuracy and specificity of the PCR reaction.

3.4.2. The identification of *GDF*8 genotypes with the use of the RFLP technique

Products of the MioClop PCR were digested with the use of the *Hpy*CH4IV (NEB, USA) restriction enzyme according to the methodology described by Clop *et al.* (2006) and Grochowska (2016). 10 µl of solution containing PCR products were mixed with 2 µl reaction mixture. The composition of the reaction mixture by Grochowska (2016) is presented in table 8.

All samples were incubated in the temperature of 37ºC for 4 hours, after which 2 µl of 6XLoading Dye Solution (Fermentas UAB, Lithuania) was added to each sample. In order to assess the results of the PCR-RFLP the electrophoresis was performed in 2% agarose gel (Prona Agarose, Spain) with the additive of Midori Green DNA Stain (Nippon Genetics Europe GmbH, Germany), at 120V, for 90 minutes. Lambda DNA/*Hind*III Plus Marker (Life Technologies, USA) was used as a marker. Gel was archived by using UV transilluminator (wavelengths of 312 nm) G:Box Chemi XR5 (Syngene, United Kingdome).

Images obtained after the electrophoresis revealed band patterns that corresponded to myostatin genotypes. The bands patterns were as follows: genotype AA (also called c.*1232AA) was represented by one single band of the length of 1,003 bp (the length of undigested PCR product). Since the enzyme cuts ACGT sequence leaving 5'- CG overhang, a mutation that changed A nucleotide to G created the restriction site recognized by *Hpy*CH4IV (NEB, USA), thus genotype GG (c.*1232GG) was represented by two bands – 270 bp and 733 bp long. AG (c.*1232AG) heterozygotes were characterized by the following bands: 1,003 bp, 270 bp and 733 bp. After analysing images and identifying genotypes, frequencies of two alleles (A and G), and 3 resulting genotypes (AA, AG, GG) were calculated.

3.4.3. The identification of *GDF*8 genotypes with the use of the SSCP technique

Products of the Hickford PCR were subjected to SSCP reaction. The separation was carried out in 8.5% polyacrylamide gel (40% Acrylamide/Bis Solution 37.5:1; BioRad, USA).

The electrophoresis was carried out in 0,5XTBE running buffer in the DCode^{TM}Universal Mutation Detection System (Biorad, USA), with the refrigerated and heating circulator (JULABO, USA) as a cooling devise

according to the methodology proposed by Grochowska (2016). Prior to the electrophoretic separation of PCR products, buffer was chilled to 14ºC. During that time, samples were prepared as follows: 0.5 µl of PCR product was mixed with loading dye (Zhou *et al.*, 2007). Samples were denaturated for 7 minutes in 95ºC, then placed on ice to cool down and loaded on the 8,5% polyacrylamide gel. Electrophoresis lasted for 21.5 hours at 380V. The next step included visualisation of the results by silver staining using Silver Stain kit (Kucharczyk Techniki Elektroforetyczne Sp.z o.o., Poland).

In order to identify myostatin alleles and genotypes revealed by SSCP method, silver stained gels were archived as pictures by using G:Box Chemi XR5 (Syngene, United Kingdome). The images obtained after the electrophoresis revealed band patterns that corresponded to myostatin genotypes. The frequencies of each genotype and allele were calculated.

3.4.4. Sequencing of genotypes identified by SSCP technique

SSCP patterns were different than those described by Hickford *et al*. (2009), therefore, samples for each genotype were sequenced by Genomed S.A. (Poland). The obtained sequences have been aligned. All sequences were compared with reference genome for sheep (Ovis aries breed Texel chromosome 2, Oar_v3.1 NCBI Reference Sequence: NC_019459.1; NCBI, 2015) by using BLAST (2015) and MUSCLE 3.8 engine (Edgar, 2014a,b). Later the results were compared to those reported by Hickford *et al*. (2009).

3.5. DATA ON LAMB MEAT PERFORMANCE

The data for Kamieniec and Pomeranian breeds was collected by the employees of the Department of Sheep and Goat Breeding, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn from the breeding documentation provided by Sheep and Goats Breeders Association members according to guidelines appropriate for the region. For those two breeds the following data was available: the body weight of a lamb in the second day of life (BW2, kg), the body weight at 56^{th} day of life (BW56, kg), sex of a lamb and type of birth (litter size indicating whether the lamb was a singleton or had a twin). Moreover, based on this data daily gains (ADG) between the second and 56^{th} day [g] were calculated.

$$\mathrm{ADG} = 1000 \times \frac{\mathrm{BW}_{56} - \mathrm{BW}_{2}}{54}$$

Where:

ADG – average daily gains between second and 56th day [g]

BW56 – body weight at the age of 56 days [kg]

BW2 – body weight at the age of 2 days [kg]

The data for Coloured Polish Merino breed was collected by The National Research Institute of Animal Production, Experimental Station in Kołuda Wielka as a part of the grant carried out in the Department of Genetics and General Animal Breeding, UTP University of Science and Technology in Bydgoszcz. This grant (N N311 521440) was founded by the Polish National Science Centre. The following information was gathered: sex of a lamb, type of birth, body weight at 2nd (BW2) [kg]. Based on the available data the weight at 30th (BW30), 56th (BW56), 70th day (BW70) [kg] and daily gains (ADG) between the second and 56th day [g] were calculated.

Moreover, for Coloured Polish Merino sheep slaughter, carcass partition and leg dissection were performed as the a part of the grant (N N311 521440) that was carried out in the Department of Genetics and General Animal Breeding, UTP University of Science and Technology in Bydgoszcz. The following measurements of different carcass traits were recorded:

1) front part of the carcass [g],
2) middle part of the carcass [g]
3) back part of the carcass [g];
4) scrag [g];
5) foreshank [g];
6) neck [g];
7) shoulder [g];
8) breast (brisket with ribs) [g];
9) entrecote [g];
10) loin [g];
11) sirloin [g];
12) hindshank [g];
13) leg [g];
14) leg prior to dissection [g];
15) muscle tissue in leg [g];
16) fat tissue in leg [g];
17) bone tissue in leg [g],
18) percentage [%] of muscle tissues in leg
19) percentage [%] of fat tissues in leg

20) percentage [%] of bone tissues in leg

Those measurements were referred to as lamb meat performance traits and the influence of different factors, i.e. myostatin genotypes, sex and type of birth on those traits was investigated.

3.6. STATISTICAL ANALYSIS

Based on the results obtained by RFLP and SSCP methods observed genotype and allele frequencies were calculated for each bread. The $\chi 2$ was used in order to investigate the differences regarding genotype frequencies between tested breeds.

The data containing information about genotypes detected by RFLP technique was further subjected to the Hardy-Weinberg equilibrium test using the programme Arlequin ver. 3.0, (Excoffier *et al*., 2005).

The tested meat performance traits within each breed were statistically characterized by calculating basic statistical measures: arithmetic mean (MEAN), standard deviation (Std) and coefficient of variation (CV). In order to investigate effect of myostatin genotypes, breed, sex and type of birth on tested traits the multi-factor analysis of variance using the least squares method was applied (GLM procedure). The linear models were created separately for RFLP and SSCP methods. The analysis was carried out in two steps: for each breed separately (Model 1, a three-factor model) and for the whole tested population (Model 2, a four-factor model). Statistical analysis was carried out with the use of statistical package SAS (SAS Inc., 2015).

Model 1.

$$y_{ijkl} = \mu + a_i + b_j + c_k + (ab)_{ij} + (ac)_{ik} + (bc)_{jk} + e_{ijkl}$$

Where:

y_{ijkl} – tested trait

μ – total average

a_i – effect of lamb's sex (male, female)

b_j – effect of lamb's type of birth (singleton, twin)

c_k – effect of myostatin genotypes detected by RFLP or SSCP

$(ab)_{ij}$,$(ac)_{ik}$,$(bc)_{jk}$ – first-degree interactions between factors

e_{ijkl} – random error

Additionally in order to expand the analysis, a second linear model was used in which the additional factor – breed – was added. Three tested populations were merged into one bigger group and then the statistical analysis was repeated by using the Model 2.

Model 2.:

$$y_{ijklm} = \mu + a_i + b_j + c_k + d_l + (ab)_{ij} + \ldots + (cd)_{kl} + e_{ijklm}$$

Where:

y_{ijklm} – tested trait

μ – total average

a_i – effect of lamb's sex (male, female)

b_j – effect of lamb's type of birth (singleton, twin)

c_k – effect of myostatin genotypes detected by RFLP or SSCP

d_l – effect of breed (Kamieniec, Pomeranian, Coloured Polish Merino)

$(ab)_{ij} + \ldots + (cd)_{kl}$ – first-degree interactions between factors,

e_{ijklm} – random error

The group of animals used to investigate the association between tested factors and carcass traits consisted solely of Coloured Polish Merino males, therefore, the linear model used during the statistical analysis contained only two factors: effect of lamb's type of birth and effect of myostatin genotypes detected by SSCP.

If the analysis shown statistical differences, the data was then subjected to post-hoc test. The Duncan's multiple range test for arithmetic means was used in order to investigate the relationship between factors and studied body weights. The statistical analysis was performed by using SAS software (SAS Inc., 2015).

During the statistical analysis some groups of genotypes with the small number of representatives were eliminated. If the statistical group had not consisted of at least five individuals, the group would have been excluded from the further analysis.

4. RESULTS

4.1. DNA ISOLATION, AMPLIFICATION, QUANTITATIVE AND QUALITATIVE ASSESSMENT

Extracted DNA was examined in terms of quality and concentration. Electrophoresis separation revealed one single band for each DNA sample, which attested to good DNA quality. The results were later confirmed by performing quantitative analysis with the use of spectrophotometer NanoDrop 2000. The ration of absorbance at 260 nm and 280 nm fluctuated between 1.7-1.9, therefore, isolation was successful and resulted with the DNA of a good quality. The data gathered during those steps was used in order to prepare DNA solutions with the concentration of 50 ng DNA/µl.

During the course of study two different PCR reactions were carried out. Products of the MioClop PCR were intended for further analysis with the use of RFLP method while the second set of PCR products (obtained by Hickford PCR) was used during SSCP. Both PCR reactions were successful which resulted from the right combination of reaction mixture compositions and adequate parameters used during amplification. PCR products obtained during amplification were tested by performing agarose gel electrophoresis. For each set of PCR products electrophoresis revealed one single band of a specific length. The electrophoresis of MioClop PCR products showed one strong band of the right length (1,003 base pairs) and the gel separation of Hickford PCR products revealed the band that consisted of 414 base pairs. The results of gel separation attested to the accuracy and specificity of the PCR reaction.

4.2. THE GENETIC STRUCTURE OF THE TESTED POPULATIONS IN RELATION TO *GDF*8 GENE

4.2.1. The frequencies of alleles and genotypes detected with the use of RFLP method

Two alleles (A and G) and three resulting genotypes (AA, AG and GG) were detected. Allele A was interchangeably called allele c.*1232A, while allele G – c.*1232G. If the mutation was present (allele A, guanine had been replaced by adenine in 3'UTR) restriction enzyme did not recognize restriction site and gel separation revealed only one single band that was of the length of undigested PCR product (1,003 bp). However, when enzyme recognized restriction site (allele G) two shorter bands were visible (270 and 733 bp). Band patterns for each genotype at the c.*1232 of myostatin gene are presented

on Image 1. By analysing the results of agarose gel electrophoresis, myostatin alleles and genotypes were identified.

The AA genotype was the least frequent. It's presence was detected in two sheep breeds: Kamieniec and Pomeranian, and it's frequency was identical (6%). Prevalence of allele AG varied between those two breeds. Most frequently AG genotype was detected in Pomeranian sheep (56%), almost two and a half times more often than in the case of Kamieniec sheep (only 21%). For Coloured Polish Merino and Kamieniec breeds the most common genotype was GG (100% and 73% respectively) (Table 9).

The results revealed that not all alleles were detected in all three tested sheep breeds. Amongst the tested population of Coloured Polish Merino only allele G was detected. In contrast, two alleles, A and G, were recorded in Kamieniec and Pomeranian breeds. The frequency of allele A was the highest in Pomeranian sheep (34%), while the frequency of this allele in Kamieniec was recorded at the level of 16.5%. The 83.5% of Kamieniec and 66% of Pomeranian sheep carried allele G (Table 10).

Since all alleles were identified only in Kamieniec and Pomeranian sheep breeds (all Coloured Polish Merino sheep were homozygous for GG mutation and thus the population was not in genetic equilibrium), therefore, Hardy-Weinberg equilibrium test was performed only for those two tested populations. The observed heterozygosities for Kamieniec and Pomeranian breeds were 0.21 and 0.56, respectively, while expected heterozygosity: 0.28 and 0.45. P value for Kamieniec breed was 0.024 and for Pomeranian 0.023, therefore, both were not consistent with Hardy–Weinberg equilibrium. Test chi-square that was performed in order to verify whether differences between frequencies were significantly different among three tested breeds revealed that the differences were indeed significant (Table 9 and 11) (SAS Inc., 2015).

4.2.2. The frequencies of alleles and genotypes detected with the use of SSCP method

The genotypes detected by SSCP technique were determined and frequency of each alleles and genotype was calculated. The study revealed 8 distinctive genotypes. In order to distinguish alleles detected during the present studies from those described by Hickford *et al.* (2009), alleles were named as follows: A_1-F_1. Image 2 presents the patterns obtained during the study. Not all genotypes were detected in all sheep breeds (Table 11). Only three were identified in all three populations: A_1B_1, A_1A_1 and A_1C_1. The frequency however, varied greatly. While genotype A_1B_1 was the most frequent in Kamieniec population (51%) in case of remaining two groups it's frequency was low (1% in Coloured Polish Merino and 8% in Pomeranian).

The genotype A_1A_1 was present is all three groups and it's frequency was high in all of them – the highest in Pomeranian (89%), Coloured Polish Merino (79.4%) and the lowest in Kamieniec (25%). This genotype was the most frequent genotype reported in Coloured Polish Merino and Pomeranian. Another genotype that was recorded in all groups was genotype A_1C_1, however, its prevalence was law – only one individual of Kamieniec, one of Pomeranian (1%) and 4 of Coloured Polish Merino carried this genotype. In Kamieniec sheep 3 genotypes had the overall frequency at the level of 96% – the genotype A_1B_1 (51%), B_1B_1 (20%) and A_1A_1 (25%), also three other genotypes were recorded in this breed: A_1C_1, B_1C_1 and A_1D_1, but their frequency was very law (1%, 2% and 1%). In Coloured Polish Merino breed 5 SSCP patters were identified: A_1B_1 (1%), A_1A_1 (77%), A_1C_1 (4%), A_1E_1 (11%) and A_1F_1 (4%). In the population of Pomeranian sheep 5 genotypes were recorded: A_1B_1 (8%), A_1A_1 (89%), A_1C_1 (1%), A_1D_1 (1%) and A_1E_1 (1%).

In all three studied breeds the most frequent was allele A_1 (54.1% in Kamieniec, almost 90% in Coloured Polish Merino and 95% in Pomeranian sheep). The frequency of other alleles varied according to breed. Allele B_1 was frequent in Kamieniec sheep (46.5%), however, in remaining two breeds it's frequency was very low (0.52% in Coloured Polish Merino and 4% in Pomeranian sheep). Other genotypes did not appear frequently. The frequencies of alleles are presented in table 12.

4.3. SEQUENCES OF SNP DETECTED IN OVINE INTRON 1 OF MYOSTATIN GENE

The obtained SSCP patterns were different from those described by Hickford *et al*. (2009), therefore, samples for each genotype detected by SSCP technique were subjected to DNA sequencing.

Sequences were compared between each other and in relation to the reference genome (NCBI Reference Sequence: NC_019459.1, Ovis aries breed Texel chromosome 2, Oar_v3.1) by using NCBI BLAST (BLAST, 2015) as well as aligned with the use of MUSCLE 3.8 engine (Edgar, 2004a,b) (Fig. 3). Later the results were compared with those reported by Hickford *et al*. (2009). Sequencing confirmed that the tested region consisted of 414 nucleotides and covered the fragment of myostatin gene starting from 346^{th} nucleotide (position 346 from the beginning of the gene) (NCBI Reference Sequence: NC_019459.1, Ovis aries breed Texel chromosome 2, Oar_v3.1). It has been found that the sequence for A_1A_1 genotype was 100% identical to the reference genome, while other sequences were similar in 99%. Eight sites were found to be polymorphic in the given sequence – starting from the beginning of myostatin gene – nucleotide at the position: 391^{th}, 474^{th}, 614^{th}, $616t^{h}$, 619^{th}, 622^{th}, 632^{h} and 696^{th}. Six alleles and resulting 8 genotypes were

found in the tested population. Fig 3 presents sequences throughout all detected alleles. The differences between respective alleles are presented in table 13 and between genotypes in table 14.

Sequencing revealed the presence of thymine at the position 391 in all alleles except for allele A_1, where thymine was replaced by guanine. The position 474 is characterized by the transition between cytosine (alleles A_1 – E_1) and thymine in allele F_1. The sequence of most alleles contained thymine in the position 614; only in the case of allele B_1 transition T>C occurred. Guanine was the 616^{th} nucleotide in the DNA sequences of alleles A_1, E_1 and F_1, the sequence of alleles B_1-D_1 was characterized by transition G>A. In position 619 thymine was detected in the case of 5 alleles, only allele D_1 revealed the presence of cytosine. Cytosine was also detected in the position of 622^{th} nucleotide, all other alleles contained thymine. Another SNP was spotted at the position 632 – transversion between guanine (allele A_1) and thymine (other alleles) was detected. The last polymorphic site, in the position 696, was characterized by the presence of cytosine, only allele C_1 revealed different nucleotide (thymine) (Table 13).

4.4. THE ASSOCIATION BETWEEN MYOSTATIN GENOTYPES DETECTED DURING RFLP ANALYSIS AND LAMBS' BODY WEIGHT MEASUREMENTS

Statistical analysis using linear Model 1 did not confirm the association between myostatin genotypes detected by RFLP and selected body weights (BW2, BW56, ADG). All three myostatin genotypes (AA, AG, GG) were identified only in two sheep breeds (Kamieniec and Pomeranian), and only those two breeds were taken into consideration while investigating the relationship between occurrence of genotypes and selected body weights. Among Kamieniec the highest body weight at 56^{th} day and daily gains were found in the group of individuals with AG genotype (18.6kg and 253.2g, respectively). The smallest daily gains were recorded for GG homozygotes (213.8g). In the group of Pomeranian sheep the tendency was reversed. The highest body weight at 56^{th} day and the highest daily gains were recorded for animals with two G alleles (21.54kg and 322g) and the lowest for AA homozygotes (20.27kg and 300.3g). All the differences however were not statistically significant. Statistical characteristic in terms of different body weights in relation to genotypes detected by RFLP technique have been presented in table 15.

Additionally, analysis was carried out with the use of the second linear model (Model 2) in which a new factor – breed – was included. It has been found that all factors (genotypes detected by RFLP, sex, type of birth and breed) had significant influence on body weight at 2^{nd}, 56^{th} day as well as on daily

gains between 2nd and 56th day of life. All three genotypes detected by RFLP technique (AA, AG, GG) were present in the tested population. The lowest body weight at 2nd day was recorded for AG heterozygotes (4.39kg). Two remaining genotypes, while higher in values, did not differ significantly. The highest body weight at 56th day was reported for animals with AG genotype (20.66kg). Both homozygotes (AA and GG) were characterized by lower values (18.78kg and 18.80kg, respectively). Animals with AG genotypes were characterized by the greatest daily gains at the level of 301.4g per day ($P \leq 0.01$) (Table 21).

4.5. THE ASSOCIATION BETWEEN MYOSTATIN GENOTYPES DETECTED DURING SSCP ANALYSIS AND LAMB MEAT PERFORMANCE TRAITS

Even though a total of 8 genotypes have been detected by SSCP technique, only few of them were taken into consideration while performing statistical analysis. If the tested group did not consist of at least five representatives it was excluded from the analysis. In case of Kamieniec sheep only three genotypes were included into statistical Model 1. The highest values for all three studied weights were obtained for individuals with A_1B_1 genotype (4.95kg at 2nd day, 17.11kg at 56th day and 225.3g gain per day) and the lowest for A_1A_1, although the differences were not statistically significant (Table 16). No statistically significant association between genotypes detected by SSCP and body weights was found while evaluating the population of Pomeranian sheep. However, it has been noticed that individuals with A_1A_1 genotype seemed to be characterized by a greater body weights at 2nd (4.18kg) and 56th day (21.61kg) as well as higher daily gains between those two days (322.9g) than individuals with A_1B_1 genotype (4.06kg, 19.9kg and 293.2g, respectively). Only those two genotypes were included into statistical Model 1 (Table 17). In case of Coloured Polish Merino lambs also two genotype groups (A_1A_1 and A_1E_1) were sufficiently numerous to be taken into consideration while creating linear Model 1. The highest values for body weights at 2nd (5.13kg) and 56th day (8.34kg) were obtained by animals with A_1E_1 genotypes, while body weight at 30th (12.28kg), 70th (23.63kg), and daily gains between 2nd and 56th day (232.2g) were the lowest for A_1A_1 homozygotes (Table 18). However, no statistical influence of myostatin genotypes on meat performance traits was proven.

Additional analysis that used linear Model 2 has taken into account 4 genotypes detected by SSCP technique: A_1B_1, B_1B_1, A_1A_1 and A_1E_1. Highly significant differences were found between those groups in terms of body weights ($P \leq 0.01$). In respect of body weight at 2nd day, the highest values were recorded for individuals with A_1E_1 genotype (5.13kg), while the lowest for

A_1A_1 (4.5kg). The tendency was reversed for body weight at 56th day. The highest body weight and the greatest daily gains were reported in the group of animals with A_1A_1 genotype (20.06kg and 287.6g/day). The lowest values were recorded for B_1B_1 genotype (16.95kg and 223.7g/day) (Table 21).

4.6. THE ASSOCIATION BETWEEN MYOSTATIN GENOTYPES AND OTHER TESTED FACTORS AND LAMBS' CARCASS TRAITS

Only Coloured Polish Merino rams were used during the statistical analysis in order to investigate the influence of different factors on carcass traits. Since the tested group was monomorphic in terms of c.*1232G>A mutation and consisted solely of males, only genotypes detected by SSCP technique and type of birth were taken into consideration. The results of this analysis are presented in tables 20a and 20b. The analysis did not reveal the statistically significant influence of genotypes detected by SSCP technique on carcass traits. It may have been caused by the fact that the population of animals used for the analysis was small and uneven in terms of the number of individuals in each of tested group.

The studies showed statistical influence of type of birth on the weight of entrecote, fat tissue in leg and percentage of fat tissue in leg. Entrecote obtained from singletons was significantly heavier (582.5g) than the one from a lamb that was born in a litter (497.3g). Singletons had also more fat tissue in their legs (284.4g) and a higher percentage of fat tissue (13.77%) than twins (208.3g and 11.53%). Overall, carcass cuts were heavier for singletons (Table 20a and 20b).

4.7. THE ASSOCIATION BETWEEN LAMB'S SEX, TYPE OF BIRTH AND BODY WEIGHT MEASUREMENTS

The statistical analysis performed separately for all three tested populations (Model 1) revealed that other factors, such as sex of the lamb, had the influence on tested body weights (Tables 19a and 19b). It has been noted that for all sheep breeds males were characterized by a greater body weight than females. The differences were highly significant ($P \leq 0.01$) or significant ($P \leq 0.05$). The type of birth (litter size) also influenced tested body weights. In all breeds weight of an individual decreased with the increasing litter size. Since these factors were not the subject of the present thesis they will not be discussed extensively (Tables 19a-19b).

After using the second, extended linear model (Model 2), breed, sex and type of birth were identified as factors affecting studied body weights (Table 22). At 2nd day of life Kamieniec and Coloured Polish Merino sheep

were significantly heavier (4.92kg and 4.86kg, respectively) than sheep of Pomeranian breed (4.17kg). The body weight at 56th day was the greatest in the group of Pomeranian sheep (21.41kg), followed by Coloured Polish Merino lambs (19.48kg), and the lowest body weight was recorded in Kamieniec group. Pomeranian lambs gained weight the quickest of all three studied populations – 319.3 g per day, while the Coloured Polish Merino gained 266.3g/day, the slowest increase of weight was recorded for Kamieniec lambs (only 223g/day). Moreover, sex and type of birth also affected selected body weights. Males and singletons were characterized by grater body weights at 2nd and 56th day as well as by the greatest daily gains. Males increased the body weight on the average of 277.3g/day, whilst females only 261.4 g/day. In terms of body weight singletons had an advantage over sheep born in a litter, at second and 56th day of their lives singletons weighted more (4.76kg, 21.49kg) than twins (4.53kg, 17.08kg), and gained weight on the average level of 309.4 g/day, while twins increased their body weight 230g/day. All of the described differences were significant or highly significant (Table 22).

5. DISCUSSION

The rapid development of techniques of molecular genetics allows scientists to investigate genetic basics of animal performance. Molecular tests are widely used to identify polymorphism in genes that are associated with quantitative traits. One of the investigated genes is myostatin located in the second ovine chromosome. The wide selection of molecular tools allows to select a test according to researchers needs and capabilities, this includes equipment availability.

5.1. MOLECULAR TOOLS USED IN MYOSTATIN GENE INVESTIGATIONS

The aim of the present study was to evaluate the relationship between polymorphism in myostatin gene and meat performance, therefore, molecular genetic tests were applied in order to determine myostatin gene polymorphisms. Three molecular techniques, namely PCR-RFLP, PCR-SSCP and sequencing were used. In order to determine the frequencies of c.*1232G>A alleles and genotypes, PCR-RFLP technique was used with the use of *Hpy*CH4IV restriction enzyme. The methodology of this test was originally described by Clop *et al.* (2006), who carried out the test in order to investigate the SNP mutation in Texel sheep. Since that time, the technique is widely used in molecular studies on myostatin gene polymorphism. For instance, Johnson *et al.* (2009) used this technique to identify c.*1232A and c.*1232G alleles in the population of New Zealand (NZ) Texel sheep. Jamshidi *et al.* (2014) used *Hae*III restriction enzyme to investigate myostatin polymorphism in Mehraban sheep. The same enzyme was used by Akbari *et al.* (2015). They applied PCR-RFLP method in order to study the polymorphism in *GDF*8 gene in Kordi sheep of Northern Khorasan. Georgieva *et al.* (2015) investigated polymorphism in myostatin gene's exon 3 in Synthetic Population Bulgarian Milk (SPBM) sheep breed. The number of studies using the RFLP technique attests to the popularity of this technique. Indeed, restriction fragments length polymorphism analysis is one of the commonly used molecular tests due to its simplicity. It does not require the purchase of new and expensive equipment. Despise having a number of advantages, RFLP technique possesses one main drawback. It cannot be used to identify new mutations (Rasmussen, 2012). Therefore, when trying to identify new polymorphic sites scientists use different methods, one of them being single-stranded conformational polymorphism (PCR-SSCP).

In the present study an attempt to detect new polymorphisms in myostatin gene was undertaken, thus PCR products were subjected to SSCP gel separation. The same technique was used by various authors. One of them being Hickford *et al.* (2009) who studied ovine myostatin gene in New Zealand (NZ)

Romney sheep. Sumantri *et al.* (2011) reported the use of SSCP method while investigating c.960delG mutation in Indonesian local sheep. The study was also undertaken to determine SSCP patterns in the Iranian Baluchi sheep (Ansary *et al.*, 2011). The number of other authors used the SSCP technique during their study on *GDF*8 polymorphism (Zhou *et al.*, 2008; Han *et al.*, 2010; Farhadian *et al.*, 2011 etc.).

Different authors investigated polymorphisms in the first intron of myostatin gene using SSCP method. However, it should be noted that they reported different SSCP patterns. The differences were caused by a few factors. First of all, not all researchers amplified the same part of intron 1. In the present study a 414-bp part of intron 1 was amplified. The same part was investigated by Hickford *et al.* (2009) and Farhadian *et al.* (2012), however, in both cases the parameters of electrophoretic separation differed. In this study samples were loaded on 8.5% polyacrylamide gel and run at 380V for 21.5 hours in 14ºC (Grochowska, 2016). Parameters differed from those applied by Hickford *et al.* (2009). They used 12% polyacrylamide gel and carried out the electrophoresis for 18h at 350V and in the temperature 12ºC. Farhadian *et al.* (2012) used different parameters: 8% polyacrylamide gel, 3h, 200V and 18ºC, therefore, the images of SSCP patters differed. Other authors reported amplifying different parts of intron 1. Zhou *et al.* (2008) amplified a fragment of *GDF*8 gene that covered a part of 5'UTR, the first coding domain and the beginning of the first intron. The parameters of SSCP separations were also different from the one suggested by other authors. The separation was carried out for 18h in 14% polyacrylamide gel at 400V and at 4ºC. Moreover, Ansary *et al.* (2011) investigated a fragment of intron 1 that consisted of 291 bp, using 8% polyacrylamide gel. The separation was run for 8h at 250v and at 6ºC. Also Dehnavi *et al.* (2012) chose to investigate different part of intron 1. They amplified a region consisting of 222 bp and separated samples on 10% gel for 4h at 10ºC and at 250V.

The third technique that has been used in the present study was sequencing. It is a very useful tool, which allows to determine the exact nucleotide sequence of the investigated DNA region. Scientific literature shows many examples of studies which used this method. For instance, Boman and Våge (2009) sequenced DNA samples in order to find mutations in Norwegian Spælsau sheep myostatin gene.

Other techniques have been used during myostatin based studies. Boman *et al.* (2011) reported using SEQUENOM MassARRAY platform for SNP genotyping, Haynes *et al.* (2013) subjected their samples to real-time PCR, while Bignell *et al.* (2009) used microsatellites (BM81124 and BULGE20) which flanked ovine myostatin gene. Moreover, there is one commercially used test detecting polymorphism in *GDF*8 sequence. The test, called MyoMAX®,

identifies c.*1232G>A mutation which is associated with the improvement of muscle weight (MyoMAX, 2016). Masri *et al.* (2011a) reported using this test in their study on c.*1232G > A mutation in Texel×Welsh Mountain lambs.

5.2. POLYMORPHISM AT THE c.*1232 POSITION OF MYOSTATIN GENE

One of the main purposes of the present study was to identify c.*1232G>A alleles and genotypes in three Polish breeds: Kamieniec, Pomeranian and Coloured Polish Merino. The c.*1232G>A mutation is characterized by the G>A transition in 3'UTR of myostatin gene and allele c.*1232A is believed to be favourable in terms of meat performance in many species. In the present study a total of 297 animals were genotyped for this mutation. It was noted that Coloured Polish Merino population was monomorphic as the frequency of GG genotype was 100%. In other two populations two alleles (A and G) and three genotypes (AA, AG, GG) were identified. The frequency of allele A was higher in Pomeranian population (34%) than in Kamieniec (16.5%). In Kamieniec sheep the most common genotype was genotype GG (73%), however, in Pomeranian population the highest frequency was recorded for heterozygous genotype (56%). This suggests that the presence of c.*1232G>A mutation and the number of alleles may be breed dependent. This is in accordance with the conclusions derived by other authors that carried out studies aiming to identify this mutation in different sheep breeds. Clop *et al.* (2006), who described this mutation for the first time, reported that the frequency of allele A is near fixation in Texel sheep population (99%) and suggested that this mutation may be specific for that breed. It was later confirmed by other authors (Kijas *et al.*, 2007; Hadjipavlou *et al.*, 2008). The frequency of allele A was also high in East Frisian population (63%) described in the paper written by Bignell *et al.* (2009). They reported that the frequency of genotypes AA, AG and GG were as follows: 36.7%, 52% and 11.3%. Trukhachev *et al.* (2015), who investigated the mutation in Dzhalginsky Merino breed, indicated that 80% of the tested population was carrying two copies of allele A and 20% two copies of allele G. Also Masri *et al.* (2011a) identified the frequency of allele A to be at a high level (53.7%) in Texel × Welsh Mountain lambs. Lower values were reported by Hadjipavlou *et al.* (2008), who investigated the presence of c.*1232A allele in Charollais sheep. The prevalence of allele A in that breed was at an intermediate level (30%). Moreover, Han *et al.* (2010) indicated that allele G was more frequent in New Zealand (NZ) Romney population (77.2%), with only 22.8% of animals carrying allele A. They also noted that no AA genotypes were identified in the tested population. Authors mentioned above reported the frequency of allele A to be at high or intermediate levels, in contrast, Han *et al.* (2015), who investigated polymorphism in *GDF*8 gene in New Zealand (NZ) Romney

sheep, indicated that only 2.79% of tested animals carried allele A. Kijas *et al.* (2007) investigated the prevalence of allele A in different sheep breeds and reported that the frequency of this allele in Lincoln, Poll Dorset and White Suffolk breeds was low (12.5%). Moreover, some authors pointed out that the allele A is not present in various sheep populations. Hadjipavlou *et al.* (2008) informed that no Suffolk sires used in the study carried allele A. Also the absence of c.*1232G>A mutation in Iranian sheep breeds (Chaal, Zandi and Zel sheep) was reported (Miar *et al.*, 2011), although it might have been caused by a small number of animal in the tested population. All things consider, the frequency of allele A, which is believed to be associated with increase in meat performance traits, differ in different sheep breeds, which confirms the theory that the prevalence of c.*1232 alleles and number of copy of each allele may be dependent on sheep breed.

In the present study both c.*1232 alleles were detected only in Kamieniec and Pomeranian populations and for those two breeds expected and observed heterozygosities were calculated. In Kamieniec population expected and observed heterozygosities (0.28 and 0.21, respectively) were lower than the one obtained for Pomeranian sheep (0.45 and 0.56). This suggest that the Pomeranian population is more diverse in terms of c.*1232 alleles and sheep of that breed are more likely to carry two different alleles than individuals of Kamieniec sheep. Both populations were tested in order to verify whether they were consistent with Hardy–Weinberg equilibrium. Both obtained P values (0.0241 and 0.0232) attested to the fact that both populations were not in Hardy–Weinberg equilibrium. Moreover, in the population of Coloured Polish Merino, all tested individuals of this breed carried two copies of allele G, therefore, population was not in genetic equilibrium. Also Bignell *et al.* (2009) checked whether their tested population was in Hardy–Weinberg equilibrium. They reported that the value for chi-square test was 0.94 (1 degree of freedom) did not allow to dismiss the null hypothesis, therefore, tested population was in agreement with the said equilibrium.

In the present studies alleles c.*1232A and c.*1232G were detected in two breeds: Kamieniec and Pomeranian. Although the frequencies were not very high for allele A, the presence of this allele may be caused by the fact that in the past ewes of both breeds were crossbred with Texel rams in order to improve meat performance traits (Kawęcka and Sikora, 2012). Since Kamieniec and Pomeranian sheep are dual-purpose breeds, the focus was not put solely on their meat performance, therefore, allele A is present in both populations at low or intermediate level. However, the number of copies of allele A may be increased by implementing appropriate breeding programs. Boman *et al*. (2011) informed that in Norwegian White Sheep population the frequency of allele A increased during the period of 16 years. They investigated the change

in occurrence of c.*1232G>A mutation in the tested population between 1990 and 2006. They reported that the frequency of allele A increased from 30% in the year 1990 to 82% in 2006 which suggested that it may be brought near fixation in the nearest future.

5.3. POLYMORPHISM IN INTRON 1 OF MYOSTATIN GENE

In the present study a 414-bp part of intron 1 of myostatin gene was amplified and subjected to SSCP separation. Eight different genotypes detected by SSCP technique and 6 alleles (A_1-F_1) were identified. Only three genotypes (A_1B_1, A_1A_1 and A_1C_1) were present in all three tested populations. In Kamieniec sheep the most frequent were genotypes A_1B_1 (51%), A_1A_1 (25%) and B_1B_1 (20%), the frequency of other genotypes did not exceed 2%. In the population of Coloured Polish Merino sheep the most frequent was A_1A_1 (79.4%), but genotypes A_1B_1, A_1C_1, A_1E_1 and A_1F_1 were also identified. In population of Pomeranian sheep 89% of animals were homozygous for allele A_1, 8% had A_1B_1 genotype. Frequencies for other genotypes (A_1C_1, A_1D_1 and A_1F_1) did not exceed 1%. In all three tested populations the most frequent was allele A_1 (51.5%, 89.69% and 94.5% in Kamieniec, Coloured Polish Merino and Pomeranian populations, respectively). In Kamieniec population the prevalence of allele B_1 was also high (46.5%), however, it's frequency in two remaining breeds was low. Other authors also investigated the polymorphisms in intron 1 of *GDF*8 gene. For instance, Hickford *et al.* (2009), who studied mutations in a fragment of intron 1, identified five alleles and named them with the use of letters A-E. They reported that in NZ Romney sheep population only three alleles (A, B and C) and 6 genotypes were detected. The frequencies of each genotypes were as follows: AA (46.6%), AB (30.2%), AC (13.3%), BB (5.8%), BC (3.5%) and CC (0.6%). The most frequent was allele A (68.3%) and the least - allele C (9%). Other author that reported the information regarding polymorphisms in the same part of intron 1 was Farhadian *et al.* (2012) who identified four genotypes: AD, AC, AE and BC which occurred with the frequency of 41.3%, 29.3%, 13% and 16.3%, respectively. The most frequent were alleles A (41.85%), C (22.83%) and D (20.65%). Intron 1 of myostatin gene was investigated also by other authors, however, some used different nomenclature when naming genotypes detected by SSCP. Zhou *et al.* (2008) described genotypes *01 (86%), *02 (12%) and *03 (2%), Han *et al.* (2015) reported the presence of 5 different alleles in the samples derived from Romney rams: H1 (2.79%), H2 (53.13%), H4 (24.55%), H5 (15.19%) and H7 (4.34%). They reported that the most frequent genotypes in the population of Romney rams were H2H2 (34.08%), H3H2 (26.78%) and H5H2 (16.34%). Different names of SSCP haplotypes were reported in the paper of Ansary *et al.* (2011). They investigated polymorphisms in 291-bp fragment of the first intron in *GDF*8 gene in Iranian Baluchi sheep.

They detected three genotyped and named them: P1, P2 and P3. The most common was P2 genotype with the frequency at the level of 56.25% and the lowest frequency had genotype P3 (18.75%). Not all authors however, identified different genotypes during SSCP analysis of first intron. For instance, Dehnavi *et al.* (2012) reported that the 222-bp part of intron 1 of *GDF*8 gene in Zel Steep was monomorphic.

5.4. SNP SEQUENCES DETECTED IN OVINE INTRON 1 OF MYOSTATIN GENE

Since both region of intron 1 and SSCP parameters differed in reports of different authors, it was necessary to sequence samples for each obtained genotype. This facilitated the comparison of the results of the present study with those obtained by other authors. In this study eight distinctive genotypes were detected, the samples for each of them were subjected to sequencing. The obtained sequences were compared with the reference genome (Oar_v3.1) and the results reported by Hickford *et al.* (2009). This comparison revealed 8 SNPs: g.391G>T, g.474C>T; g.614T>C; g.616G>A; g.619T>C; g.622T>C; g.632G>T; g.696C>T. The sequences of detected SNP for all alleles and genotypes are presented in tables 13 and 14. Alleles E_1 and F_1 were not detected in Kamieniec breed, but both were present in Coloured Polish Merino population. In Pomeranian population allele F_1 and in Coloured Polish Merino allele D_1 were not detected. In the present study g.474C>T mutation was detected only in animals with A_1F_1 genotype, therefore, this mutation was not detected in Kamieniec and Pomeranian breeds. Another SNP, g.619T>C, was spotted only in animals with A_1D_1 genotype, thus it was not detected in tested Coloured Polish Merino population. Other SNPs were present in all three populations. The results of the present study confirm the presence of SNP in intron 1 that were mentioned previously by other authors. One of the first to describe some of these mutations were Clop *et al.* (2006) who tried to identified SNPs in Texel myostatin gene. Their team identified 7 SNPs in intron 1 that correspond to mutations detected in the present study, however, they did not detect g.614T>C mutation. Hickford *et al.* (2009) did identify g.614T>C in NZ Romney sheep along with 7 other mutations. They detected 5 alleles (A-E) and identified nucleotide sequence in each SNP site. It should be noted that the sequences for all alleles were similar with the ones obtained in the present study except for the SNP g.474C>T. While Hickford *et al.* (2009) reported that for all alleles the detected nucleotide in the position 474 was thymine, the results of the present study indicated that in alleles A_1 – E_1 cytosine was present. The sequence in allele F_1 was the same as the sequence of Hickford's allele E (Table 13). In order to check whether the presence of different SNPs was dependant on sheep breed Han *et al.* (2013) carried out the study on different breeds (Romney, Coopworth, Corriedale, Dorper,

Perendale, Suffolk, Merino, Dorset Down, Poll Dorset, Texel and other NZ cross-bred sheep). They detected 11 SNPs in intron 1, seven of them being at the same positions that SNPs detected in the present study. Han *et al.* (2013) did not however detect g.474C>T in neither of tested populations. They informed that no mutations were detected in Texel sheep, c.373+18T>G (at 391th myostatin gene nucleotide) was not detected in Perendale sheep; c.373+241C>T (614th nt) and c.373+243A>G (616th nt) were present in all tested populations except for Texel; c.373+246C>T mutation (619th nt) was recorded for Corriedale, Dorper, Dorset Down and NZ cross-bred populations. Another mutation, c.373+249C>T (622th nt), was identified in Coopworth, Corriedale, Coop dale, NZ Romney, Poll Dorset and NZ cross-bred sheep. All populations except for Texel and Perendale carried c.373+259T>G (632th nt) mutation, while c.373+323T>C (696th nt) was detected only in NZ cross-bred sheep. Sjakste *et al.* (2011) sequenced the part of intron 1 in animals of Latvian Darkhead sheep. They reported presence of 4 SNPs: c.373+18T>G (391th nt), c.373+241C>T (614th nt), c.373+243A>G (616th nt), c.373+259T>G (632th nt) and two not investigated in the present study: c.373+434N>A, c.373+435C>A. Another author who reported presence of different SNPs in myostatin intron 1 is Trukhachev *et al.* (2015). Intron one of Russian Dzhalginsky Merino myostatin gene was investigated and the following mutations were detected: c.373+18, c.373+241, c.373+243, c.373+259, c.373+563. Trukhachev and his team did not identify three mutations (g.474C>T, g.622T>C and g.696C>T) detected in the present study. All those studies indicate that the presence of mutations in *GDF*8 intron 1 is breed-dependant.

5.5. THE ASSOCIATION BETWEEN c.*1232 POLYMORPHISM IN MYOSTATIN GENE AND BODY WEIGHTS

In the present study the association between c.*1232 alleles and genotypes and meat performance traits was investigated. It was noted that, when all three sheep populations were analyzed as a one group (linear Model 2), c.*1232 genotypes had a statistically significant impact on body weight at 2nd (BW2) and 56th day of life (BW56) as well as on daily gains (ADG). The highest BW2 had lambs with GG genotype. Those finding are in contrast to information published by various authors who suggested that allele A had an additive effect on birth weight (Han *et al.*, 2010; Haynes *et al.*, 2013; Han *et al.*, 2015). However, other studies did not find statistically significant effect of A allele on this trait in NZ Texel cross sheep (Johnson *et al.*, 2009) or in Charollais sheep (Hadjipavlou *et al.*, 2008).

The highest BW56 and ADG were recorded for animals with heterozygous genotypes, while lambs with two copies of G alleles had the lowest values for both measurements. This is in agreement with reports of other authors

who indicated that the presence of allele A improves meat traits. For instance, Han *et al.* (2015) reported that lambs carrying allele A were characterized by increased total muscle yield, loin, shoulder and leg yields, however, Han *et al.* (2010) did not find effect on shoulder and leg yield. Hadjipavlou *et al.* (2008) indicated the improvement of muscle depth in Charollais sheep by 14%. Haynes *et al.* (2013) reported that for the population of Poll Dorset cross White Suffolk cross Border Leicester cross Merino, lams with two copies of allele A were characterized by a greater dressing percentages, shortloin, topside and round weights. Those lambs had less fat and higher carcass and meat yield compared to animals homozygous for allele G. Masri *et al.* (2011a), who tested the population of Texel × Welsh Mountain lambs, indicated the association between AA genotype and significant increase in carcass width across shoulders, breast and hind legs. They also noticed that the animals carrying only one copy of allele A did not differ significantly from animals with two G alleles. Masri *et al.* (2011b) who tested Texel and Poll Dorset rams found out that animals homozygous for c.*1232G>A mutation were characterized by the increase (by 2.8%) in loin depth, had higher lean weight, muscle to bone ratio as well as muscle to fat ratio. An increase in muscle density and decrease in intramuscular fat in animals carrying two A alleles was also noticed. Moreover, Hope *et al.* (2013) reported that AA genotype positively affected carcass yield, they also noted that the intramuscular fat content decreased in those animals. Johnson *et al.* (2009), while reporting that allele A was favourable in terms of muscling and fat content in New Zealand Texel-cross Sheep, did not find significant interactions between the number of carried copies of A allele and carcass weights. Hadjipavlou *et al.* (2008), who also investigated the influence of c.*1232G>A on meat performance traits, reported that the allele A had an additive effect on muscle depth. Another report on effects of mutation in 3'UTR of myostatin gene was presented by Trukhachev *et al.* (2015), who found that carcass length of Dzhalginsky Merino sheep that carried the mutation was greater by 2.2% than non-carriers. The presence of allele A in Australian Texels was associated with the increased in muscularity and decreased in fatness (Kijas *et al.*, 2007).

5.6. ASSOCIATION BETWEEN MUTATIONS IN INTRON 1 OF *GDF*8 GENE AND MEAT PERFORMANCE TRAITS

The association between myostatin genotypes in intron 1 and meat performance traits was investigated. The highest value for BW2 was recorded for animals with A_1E_1 genotype, however, for BW56 and ADG values were significantly higher for A_1A_1 animals. The lowest BW56 and ADG characterized sheep with two copies of allele B_1. No association was detected between those genotypes and carcass traits, which may be cause by the fact that

the tested population was not numerous since the information on carcass traits was collected only from Coloured Polish Merino rams. The association between different alleles and genotype and various measurements was reported also by Hickford *et al.* (2009). They noted that a copy of allele A was linked with the decrease in total yield, leg and loin yields. Animals carrying allele B were characterized by greater values for loin yield and proportion loin yield. They also noticed that animals homozygous for allele B had a decreased in proportion of shoulder yield. Moreover, no association between those genotypes and birth weights was proven. Another study on the influence of different SNP in intron 1 on different traits was carried out by Farhadian *et al.* (2012). They reported that in Iranian Makoei breed amongst four identified genotypes only one (AD) was associated with birth weight. No relationships between genotypes and weight gains were identified. Ansary *et al.* (2011), who identified three genotypes in intron 1 (P1, P2 and P3) reported statistically significant association between observed polymorphisms and weaning weight. Animals with P1 genotype were characterized by the highest birth, weaning weights and weight at six-months. Trukhachev *et al.* (2015) reported that animals carrying two copies of c.373+18G>T (g.391G>T) mutation significantly differed from non-carriers in terms of length of croup, loin width, width of back, half girth of back and metatarsus length. In turn, Sjakste *et al.* (2011) informed that c.373+18G>T mutation had an influence on the pre-mRNA secondary structure as T>G transition may be responsible for generating the additional hairpin and interhairpin boundary and thus result in thermodynamically stable pre-mRNA secondary structure. Authors suggested that the c.373+18G sequence may increase transcription as well as affectivity of splicing, therefore, in terms of breeding programmes, more favourable would be c.373+18T sequence.

5.7. THE INFLUENCE OF DIFFERENT FACTORS ON MEAT PERFORMANCE TRAITS

During the present study the influence of different factors on performance traits was investigated. For all three tested populations, Kamieniec, Pomeranian and Coloured Polish Merino sheep, it has been found that type of birth (litter size) had a great influence on studied measurements. Lambs that were born as singles (singletons) were characterized by grater values for BW2, BW56 and ADG, and in case of Merino sheep also BW30 and BW70. The results are in accordance with the common belief that the body weight decreases with the increase of litter size (Gardner *et al*., 2007; Chniter *et al*., 2011; Simeonova *et al*., 2014 etc.). Some authors suggest that litter size has the greatest impact on live weight (Gardner *et al*., 2007). Ivanova and Raicheva (2009, cited after Simeonova *et al*., 2014) reported that twins of Blackhead Pleven Sheep were smaller at birth and at weaning than singletons. Cloete *et al*.

(2007) suggested that since twins had to compete against each other for mother's milk, they were characterized by lower weight at weaning than singletons. Simeonova *et al*. (2014) also reported that singles gained weight faster than twins and were heavier at weaning. At the same time they pointed out that the difference in values for different live weights may be caused by the difference in birth weights.

The present study also associated the type of birth with the weight of carcass traits. It has been found that litter size has a significant influence on entrecote weight. The weight of this carcass cut obtained from singles was greater by 85g. Litter size also seemed to influence tissue composition. For instance, singletons were characterized by grater values for fat content in leg ($P<0,01$) by the average of 76g, also fat percentage in leg was higher for singletons. Other authors also reported higher carcass yield for single born individuals (Ekiz *et al*., 2012). In contrast, Simeonova *et al*. (2014) did not found any significant differences between fat content in the carcass of single and twin lambs.

The results of the present study suggest that another factor that had an impact on sheep body weights was sex of a tested lamb. It was noted that males of Kamieniec and Coloured Polish Merino sheep had a greater body weight at second and 56^{th} day of life and their daily gains were higher ($P<0,01$) than those recorded for females. Pomeranian males were also heavier at birth and at 56^{th} day of life, however, the second measurement was not proven to be statistically significant. No significant differences were found between ADG of male and female, although, the average values for females were higher. However, in overall males tended to have a greater body weight at 2^{nd}, 56^{th} day and for Coloured Polish Merino also at 30^{th} and 70^{th} day. The results are in accordance to other studies reported in various scientific magazines. Gardner *et al*. (2007) investigated the influence of different factors on birth weight of Blue-faced Leicester×Swaledale and Welsh Mountain ewes. They stated that sex of lamb had a great influence on the tested weight since males were 363g heavier than females. Also Daskiran *et al*. (2010) and Simeonova *et al*. (2014) noted that male lambs gained weight faster than females. However, according to Manso *et al*. (1996) females, even though born with lower body weight, gain weight faster than males. This is in accordance with the results obtained in the present study for Pomeranian ewes which were characterized by higher ADG than males (although no statistical difference was found).

6. SUMMARY AND CONCLUSIONS

Based on the results obtained during the present study the following conclusions can be made:

- The extracted DNA was of a good quality, as shown by the results of electrophoretic separation and spectrophotometric measurements. The testing methods used during the present study allowed to identify the polymorphism in *GDF*8 gene at the c.*1232 position and in the fragment of first intron. The prevalence of different mutations in intron 1 was confirmed by sequencing.
- In the populations of Kamieniec and Pomeranian sheep two alleles (A and G) and three genotypes (AA, AG, GG) were detected within the *GDF*8 gene by using PCR-RFLP technique, while in the population of Coloured Polish Merino only allele G was identified. The genotype and allele frequencies varied depending on tested breed. The highest frequencies in the population of Kamieniec and Coloured Polish Merino were recorded for genotype GG, while in the population of Kamieniec sheep the most frequent was AG genotype. In terms of c.*1232G>A polymorphism all tested populations were not consistent with Hardy-Weinberg equilibrium.
- During the study eight distinctive genotypes detected by SSCP method (A_1A_1, A_1B_1, A_1C_1, A_1D_1, A_1E_1, A_1F_1, B_1B_1, B_1C_1) and six alleles ($A_1 – F_1$) were recorded. The prevalence of different genotypes and alleles as well as their frequencies varied depending on sheep breed. The most frequent in the group of Kamieniec sheep was genotype A_1B_1, however, it's frequency was very low in remaining two breeds. The highest frequency in the population of Pomeranian and Coloured Polish Merino sheep were recorded for B_1B_1 genotype. The prevalence of genotype A_1F_1 was noted only in the population of Coloured Polish Merino.
- Sequencing of the 414-bp fragment of intron 1 enabled detection a total of eight different polymorphic sites: g.391G>T, g.474C>T; g.614T>C; g.616G>A; g.619T>C; g.622T>C; g.632G>T; g.696C>T. Some minor differences between the races were present – mutation g.474C>T was detected only in the population of Coloured Polish Merino, and g.619T>C was present only in the population of Kamieniec and Pomeranian sheep.
- Analyzing, in each breed separately, the association between polymorphisms within *GDF*8 gene (detected by RFLP and SSCP methods) and lamb body weights, no statistically significant effect of the studied genotypes on these traits was proven. After including the breed factor into the analysis of variance the statistically significant impact of these polymorphisms on body weight at 2^{nd} and 56^{th} day of lamb's life as well as on daily gains between 2^{nd} and 56^{th} day was confirmed. In case of genotypes detected by RFLP technique, lambs with AG genotype were characterized

by the highest body weights at 56^{th} day and average daily gains between 2^{nd} and 56^{th} day. For genotypes indentified by SSCP, the highest values for body weight at 56^{th} day of life was recorded for lambs with A_1A_1 genotype.

- In terms of carcass traits, no statistically significant effect of genotypes detected by SSCP was reported.
- In order to expand the analysis of the association between polymorphism in *GDF*8 gene and meat performance traits, it is suggested to perform the analysis with the use of a bigger and more racially diverse population.

7. SUMMARY

The aim of the present study was to investigate the polymorphisms in two different fragments of ovine myostatin gene as well as to evaluate the frequencies of *GDF*8 alleles and genotypes in three Polish sheep breeds: Kamieniec, Pomeranian and Polish Coloured Polish Merino. Moreover, the association between myostatin genotypes and different meat performance traits (*inter alia* body weight at different days of lamb's life, average daily gains between second and 56th day as well as some carcass traits) was determined.

The studied material consisted of 297 individuals of three tested sheep breeds. Kamieniec and Pomeranian populations were composed of 100 lambs, Coloured Polish Merino population consisted of 97 individuals. In order to investigate polymorphisms in myostatin gene three different molecular techniques were used: PCR-RFLP, PCR-SSCP and sequencing. For the purpose of detecting c.*1232G>A mutation in 3'UTR of *GDF*8 gene *Hpy*CH4(IV) restriction enzyme was used (Clop *et al.*, 2006; Grochowska, 2016). Moreover, a 414-bp fragment of intron 1 was amplified and subsequently subjected to SSCP separation (Hickford *et al.*, 2009; Grochowska, 2016). The samples representing all genotypes detected by SSCP were sequenced.

The results revealed that c.*1232 genotypes varied significantly depending on breed. Coloured Polish Merino tested population was monomorph in terms of c.*1232G allele, however, two alleles (A and G) constituting three genotypes (AA, AG, GG) were detected in Kamieniec and Pomeranian breeds. The SSCP analysis of polymorphism in intron 1 of ovine myostatin gene revealed eight distinctive genotypes and six alleles. Samples for each genotype detected by SSCP were sequenced. Within the tested fragment of intron 1 eight different polymorphic sites were identified: g.391G>T; g.474C>T; g.614T>C; g.616G>A; g.619T>C; g.622T>C; g.632G>T; g.696C>T.

The statistical influence of c.*1232 genotypes on meat performance traits was investigated. It was found that animals with AG genotype were characterized by the greatest values of body weight at 56th day and daily gains. Statistical analysis revealed significant association between genotypes detected by SSCP and body weights. The highest values for body weight at 56th day and average daily gains were recorded for A_1A_1 genotype and the lowest for B_1B_1. No statistical association was found between myostatin genotypes and carcass traits.

8. STRESZCZENIE

Celem niniejszej pracy była identyfikacja polimorfizmów w dwóch fragmentach genu miostatyny oraz określenie frekwencji alleli i genotypów trzech ras owiec: kamienieckiej, pomorskiej oraz merynosa barwnego. Ponadto określono powiązania pomiędzy polimorfizmem w obrębie genu *GDF*8 a cechami użytkowości mięsnej (w tym wpływ na masy ciała w różnych dniach życia jagnięcia, przyrosty dobowe pomiędzy drugim a 56. dniem życia oraz na różne cechy poubojowe).

Badany materiał obejmował 297 osobników należących do trzech ras owiec. Populacje owiec kamienieckich i pomorskich składały się ze 100 osobników, a populacja merynosa barwnego z 97. W celu zbadania wpływu polimorfizmu w obrębie genu miostatyny wykorzystano trzy różne techniki genetyki molekularnej: PCR-RFLP, PCR-SSCP oraz sekwencjonowanie. Aby zidentyfikować mutację c.*1232G>A w 3'UTR użyto enzymu restrykcyjnego *Hpy*CH4(IV) według metodyki Clop i in. (2006) oraz Grochowskiej (2016). Z kolei mutacje w obrębie pierwszego intronu (fragment o długości 414 par zasad) zbadano dzięki zastosowaniu techniki SSCP (Hickford i in., 2009; Grochowska, 2016). Próbki reprezentujące każdy z uzyskanych wzorców SSCP zsekwencjonowano.

Uzyskane wyniki wskazują, że frekwencje genotypów c.*1232G>A były statystycznie istotnie zróżnicowane między rasami. Wykazano brak obecności allela G w obrębie badanej grupy merynosa barwnego. Jednakże dwa allele (A, G), tworzące trzy genotypy (AA, AG, GG), zostały wykryte w pozostałych dwóch rasach. Analiza intronu 1 za pomocą techniki SSCP wykazała obecność ośmiu genotypów i 6 alleli. Sekwencjonowanie próbek reprezentujących każdy wzór ujawniło obecność ośmiu miejsc polimorficznych. Wykryto następujące mutacje: g.391G>T; g.474C>T; g.614T>C; g.616G>A; g.619T>C; g.622T>C; g.632G>T; g.696C>T.

Analiza statystyczna wpływu poszczególnych genotypów c.*1232 na cechy użytkowości mięsnej wykazała, że zwierzęta posiadające genotyp AG charakteryzowały się najwyższą masą ciała w 56. dniu życia oraz najwyższymi przyrostami masy ciała pomiędzy 2. a 56. dniem życia. W przypadku genotypów określonych metodą SSCP statystycznie najwyższe masy ciała w 56. dniu życia oraz przyrosty masy ciała zostały odnotowane dla zwierząt o genotypie A_1A_1, a najniższe dla nosicieli dwóch alleli B_1. Nie wykazano statystycznie istotnych zależności pomiędzy genotypami miostatyny a cechami poubojowymi.

9. ROZSZERZONE STRESZCZENIE

9.1. Wstęp i cel pracy

Pomimo niezaprzeczalnych właściwości prozdrowotnych jagnięciny (Milewski, 2006) w Polsce nie odnotowuje się dużego zapotrzebowania na ten rodzaj mięsa. Po przystąpieniu Polski do Unii Europejskiej, powstała możliwość eksportowania polskiego mięsa na rynki zachodnie (Rokicki, 2008), dlatego tak ważne jest, aby mięso polskich owiec było konkurencyjne zarówno pod względem ceny jak i jakości.

W dzisiejszych czasach proces genetycznego doskonalenia populacji owiec może zostać wsparty przez zastosowanie nowoczesnych technik genetyki molekularnej. W celu zidentyfikowania różnych mutacji stosuje się wiele technik, między innymi PCR-RFLP, PCR-SSCP oraz sekwencjonowanie. Jednym z genów, które powiązane są z cechami użytkowymi mięsnej jest gen miostatyny, będący negatywnym regulatorem wzrostu mięśni szkieletowych. Każdy polimorfizm w obrębie tego genu, który powodować będzie zmianę sekwencji nukleotydowej, może mieć wpływ na konformację lub koncentrację produkowanego białka, a w konsekwencji może skutkować zwiększeniem masy mięśniowej zwierzęcia.

Celem niniejszej pracy była identyfikacja polimorfizmów w dwóch fragmentach genu miostatyny oraz określenie frekwencji alleli i genotypów w trzech populacjach owiec: kamienieckiej, pomorskiej oraz merynosa barwnego. Ponadto określono wpływ polimorfizmu w obrębie genu *GDF*8 na masy ciała jagniąt w różnym wieku, przyrosty dobowe pomiędzy drugim a pięćdziesiątym szóstym dniem życia oraz na różne cechy użytkowości rzeźnej określone poubojowo.

9.2. Przegląd literatury

W Polsce po roku 1986 odnotowano znaczy spadek pogłowia owiec. W celu zwiększenia konkurencyjności i dochodowości hodowcy owiec zdecydowali się zmienić ukierunkowanie swojej produkcji na mięsną (Rokicki, 2008).

W czerwcu 2015 roku liczba owiec w Polsce wynosiła 227,5 tysięcy (GUS, 2015). Tabela 1. przedstawia zmiany w liczebności populacji owiec oraz liczby maciorek na przestrzeni ostatnich lat, podczas gdy wykres 1. ukazuje zmiany liczebności populacji owiec w Polsce w latach 2003-2015. Pogłowie owiec w Polsce jest zróżnicowane rasowo. Spośród dostępnych ras hodowanych w Polsce w celu przeprowadzenia niniejszych badań wybrano trzy następujące

rasy: owcę kamieniecką, pomorską i merynosa barwnego. Tabela 2. przedstawia liczbę owiec tych ras, które zostały włączone do programu ochrony zasobów genetycznych na rok 2013.

Owca kamieniecka jest jedną z długowełnistych owiec występujących na terenie Polski. Obecnie owce kamienieckie są hodowane ze względu na mięso. Charakteryzują się dobrą wydajnością mleczną i mięsną (Lipecka i Gruszecki, 2006a). Owca pomorska to kolejna owca odmiany długowełnistej. Prace hodowlane prowadzone w obrębie tej rasy polepszyły wydajność mięsną oraz jakość wełny (Laudowicz, 1977; Baranowski, 1998, Lipecka i Gruszecki, 2006b). Polski merynos barwny to trzecia rasa wykorzystana w badaniach własnych. Dzięki podjętym działaniom hodowlanym polepszono wydajność mięsną oraz wytworzono rasę z naturalnie kolorową skórą i wełną (Pakulski, 2010; Kawęcka i Sikora, 2012). Tabela 3. przedstawia informacje dotyczące cech charakteryzujących owce kamienieckie, pomorskie i merynosa barwnego.

Identyfikacja QTL (quantitative trait loci – loci cech ilościowych) jest ważna z hodowlanego punktu widzenia ze względu na możliwość ustalenia genetycznych podstaw pożądanych fenotypów (Hartl i Ruvolo, 2012). Jak wykazały badania, QTL powiązanym z fenotypem podwójnego umięśnienia jest gen kodujący białko zwane miostatyną (Lee, 2004; Boman i in., 2009). Gen ten zlokalizowany jest w chromosomie 2 (Boman i in., 2009), a białko pełni rolę negatywnego regulatora wzrostu mięśni szkieletowych (Lee, 2004). Po raz pierwszy gen *GDF*8 opisany został przez McPherron i jego zespół w 1997 roku. U myszy pozbawionych tego genu stwierdzono znaczny przyrost mięśni.

Gen kodujący białko miostatynę zwany jest *GDF*8 (Growth Differentiation Factor 8) bądź *MSTN*. Zlokalizowany jest w drugim chromosomie, składa się z 3 eksonów i 2 intronów, koduje białko składające się z 375 aminokwasów (Boman i in., 2009). Struktura genu przedstawiona została na schemacie 1., podczas gdy schemat 2. przedstawia budowę kodowanego przez gen białka. Miostatyna syntetyzowana jest głównie w mięśniach szkieletowych (Kambadur i in., 1997; Boman i in., 2009). Białko prekursorowe składa się z 375 aminokwasów, jednakże jedynie 109 wchodzi w skład dojrzałego białka (Tellam i in., 2012).

Polimorfizm w obrębie genu kodującego miostatynę został szeroko opisany w literaturze ze względu na jego powiązanie z cechami użytkowości mięsnej. Najszerzej opisuje się polimorfizm u bydła, zwłaszcza bydła błękitnego belgijskiego, które znane jest ze swojej masywnej budowy. U tej i innych ras wykryto szereg mutacji, które powiązano ze zmieniającym się umięśnieniem. Jedną z opisanych mutacji jest nt821 (del11), czyli delecja 11 nukleotydów w eksonie trzecim, która powoduje powstanie przedwczesnego kodonu STOP. Mutację tę wykryto u następujących ras: blonde d'aquitaine, limousine,

parthenaise, rubia gallega, red angus (Karim i in., 2000; Wiener i Gutierrez-Gil, 2009; Allais i in., 2010). Kolejną mutacją jest nt419 (del7-ins10), która charakteryzuje się delecją 7 z jednoczesną insercją 10 nukleotydów, którą wykryto u bydła maine-anjou (Grobet i in., 1998). Mutacja C313Y (bydło piedmontese, gasconne) charakteryzuje się tranzycją G>A w pozycji 938. nukleotydu, powoduje ona utratę biologicznej aktywności białka (Kambadur i in., 1997; Grobet i in., 1998; Karim i in., 2000; Bellinge i in., 2005). U bydła rasy charolaise wykryto tranzycję C>T (mutacja Q204X) (Karim i in., 2000; Allais i in., 2010). Z kolei w populacji maine-anjou odnotowano wystąpienie mutacji E226X (tranzycja G>T w pozycji 676. nukleotydu) (Grobet i in., 1998; Karim i in., 2000).

Również badania nad owcami przeprowadzono w celu identyfikacji polimorfizmów występujących w obrębie genu *GDF*8. Jedną z częściej opisywanych mutacji jest c.*1232G>A, która powoduje zmniejszenie stężenia cyrkulującej we krwi miostatyny nawet do jednej trzeciej. Mutacja ta charakteryzuje się tranzycją G>A w 3'UTR (Clop i in., 2006; Boman i in., 2010; Georges, 2010). Allel A został powiązany ze zwiększeniem masy mięśniowej (Han i in., 2010). Mutacja ta została opisana u takich ras jak: texel, australian texel, poll dorset, east friesian i charollais (Kijas i in., 2007; Hadjipavlou i in., 2008; Bignell i in., 2009; Johnson i in., 2009). W obrębie rasy owcy białej norweskiej zidentyfikowano mutację c.960delG, która charakteryzuje się delecją guaniny w pozycji 960. nukleotydu. Mutacja ta została powiązana ze zwiększaniem się masy ciała zwierząt (Boman i in., 2009). Kolejną mutacją opisaną w populacji owiec jest c.120insA, czyli insercja adeniny w pozycji 120. nukleotydu (Boman i Vage, 2009). W 2009 roku Hickford i jego zespół opisali badania nad nowozelandzką owcą romney, w których za pomocą techniki SSCP wykryli 5 wzorców (A, B, C, D oraz E). Ich badania wiązały wystąpienie allela A ze zmniejszeniem się tkanki mięśniowej, a allela B z jej zwiększeniem (Hickford i in., 2009). Z kolei Ansary i in. (2011) badający irańską rasę baluchi opisali wystąpienie 3 genotypów (P1, P2, P3).

Na użytkowość mięsną zwierząt wpływa wiele czynników. Oprócz uwarunkowań genetycznych, takich jak np. rasa, znaczenie mają czynniki środowiskowe, pobierany pokarm, a nawet rok urodzenia. Powszechnie wiadomym jest, że dymorfizm płciowy wpływa na uzyskane masy, jako że samce zazwyczaj są większe od samic. Typ urodzenia również ma znaczenie, gdyż jedynaki zazwyczaj osiągają lepsze wyniki podczas ważenia niż osobniki urodzone w miocie (Piwczyński i Mroczkowski, 2005; Gardner i in., 2007; Guerrero i in., 2013).

Dokonania genetyki molekularnej pozwalają na dokładną analizę materiału genetycznego zwierząt i identyfikację osobników o pożądanym genotypie.

Technikami pozwalającymi na identyfikację alleli i genotypów są między innymi PCR-RFLP i PCR-SSCP (Awole Adem, 2006; Słomski i in., 2008). RFLP (Restriction Fragments Length Polymorphism) to technika pozwalająca na wykrycie polimorfizmów w obrębie DNA za pomocą enzymu restrykcyjnego (Kakavas i in., 2008; Słomski i in., 2008). Kolejną techniką, którą można wykorzystać podczas badania materiału genetycznego pod kątem wystąpienia polimorfizmu jest SSCP (Single Strand Conformation Polymorphism). Pozwala ona na przeskanowanie znacznej liczby zwierząt w poszukiwaniu znanych mutacji, jak i w celu identyfikacji nowych (Orita i in., 1989; Kakavas i in., 2008). Technika ta opiera się na fakcie, iż w warunkach natywnej elektroforezy pojedyncze nici DNA przyjmują strukturę trzeciorzędową, która zależna jest od sekwencji nukleotydów. Mutacje powodujące zmiany w sekwencji mogą powodować zmiany konformacji, a co za tym idzie takie fragmenty DNA będą migrować w żelu z różną prędkością (Charon i Świtoński, 2006; Kakavas i in., 2008; Słomski i in., 2008).

9.3. Materiał i metody

Badaniami objęto populację owiec składającą się z 297 osobników należących do trzech ras: owcy kamienieckiej, owcy pomorskiej oraz polskiego merynosa barwnego. Liczbę owiec objętych badaniami z uwzględnieniem rasy, płci oraz typu urodzenia przedstawiono w tabeli 4. Badane owce pomorskie pochodziły z terenu województwa pomorskiego niedaleko Łeby, owce kamienieckie wchodziły w skład stada Stacji Produkcyjno Eksperymentalnej w Bałcynach (województwo warmińsko-mazurskie). Materiał biologiczny od owcy pomorskiej i kamienieckiej udostępniony został przez pracowników Katedry Hodowli Owiec i Kóz Wydziału Bioinżynierii Zwierząt Uniwersytetu Warmińsko-Mazurskiego w Olsztynie. Stado merynosa barwnego zlokalizowane było w województwie kujawsko-pomorskim w Zakładzie Doświadczalnym Instytutu Zootechniki Państwowego Instytutu Badawczego w Kołudzie Wielkiej.

Materiał do badań stanowiła krew obwodowa pobrana w 2012 roku przez lekarza weterynarii. Genomowe DNA zostało wyekstrahowane za pomocą komercyjnego zestawu MasterPure™ DNA Purification Kit for Blood (Epicentre Biotechnologies, USA) według metodyki producenta z modyfikacjami (Grochowska, 2016). Ocena jakościowa i ilościowa uzyskanego DNA przeprowadzona została z wykorzystaniem spektrofotometru NanoDrop 2000 (Thermo Scientific, USA). Następnie DNA zostało rozcieńczone w celu uzyskania stężenia 50 ng/µl. Próbki sprawdzono również na 2% żelu agarozowym (Prona Agarose, Hiszpania) z dodatkiem Midori Green DNA Stain (Nippon Genetics Europe GmbH). Próbki poddano

elektroforetycznemu rozdziałowi, który trwał 60 minut (120V), w buforze 1XTBE (10xTBE: 0.89M Tris, 0.89M boric acid, 0.02M EDTA, pH 8.0).

Amplifikacja fragmentów genu miostatyny przeprowadzona została z wykorzystaniem dwóch par starterów w oddzielnych reakcjach PCR. Reakcje te zostały nazwane MioClop i Hickford PCR. Tabela 5. przedstawia informacje dotyczące poszczególnych starterów, a tabela 6. informacje o składzie mieszaniny reakcyjnej (Grochowska, 2016). Obie reakcje przeprowadzone zostały w termocyklerze Mastercycler pro S thermocycles (Eppendorf, Niemcy) według metodyki opracowanej przez Grochowską (2016) (Tabela 7). Po amplifikacji poszczególnych fragmentów DNA przeprowadzona została analiza jakościowa z wykorzystaniem rozdziału elektroforetycznego. Próbki zostały naniesione na 2% żel agarozowy (Prona Agarose, Hiszpania) z dodatkiem Midori Green DNA Stain (Nippon Genetics Europe GmbH). Rozdział trwał 60 minut przy 120V w buforze 1XTBE (10xTBE: 0.89M Tris, 0.89M boric acid, 0.02M EDTA, pH 8.0). Wizualizacja wyników pozwoliła na określenie skuteczności reakcji PCR. W przypadku MioClop PCR rozdział elektroforetyczny wykazał obecność fragmentu DNA o długości 1003 pz, a w przypadku Hickford PCR 414 pz.

Próbki DNA uzyskane w reakcji MioClop PCR zostały poddane trawieniu enzymatycznemu z wykorzystaniem *Hpy*CH4IV (NEB, USA) według metodyk Clop'a i in. (2006) oraz Grochowskiej (2016). Skład mieszaniny reakcyjnej zawierającej enzym przedstawiony został w tabeli 8. Próbki inkubowano w temperaturze 37ºC przez 4 godziny, po czym poddano je rozdziałowi elektroforetycznemu w celu zidentyfikowania alleli i genotypów. Uzyskany obraz prążków został zinterpretowany następująco: allel A reprezentowany był przez jeden prążek o długości 1003 pz, allel G reprezentowany był zaś przez dwa prążki (o długości 270 i 733 pz). Enzym *Hpy*CH4IV (NEB, USA) tnie DNA w miejscu o sekwencji ACGT, dlatego też zmiana nukleotydu A na G (allel G) powoduje wytworzenie się miejsca docelowego dla enzymu.

Próbki uzyskane w reakcji Hickford PCR poddane zostały rozdziałowi z wykorzystaniem reakcji SSCP. Rozdział przeprowadzony został w 8,5% żelu poliakrylamidowym (40% Acrylamide/Bis Solution 37.5:1; BioRad). Reakcję przeprowadzono z wykorzystaniem sprzętu DCode™Universal Mutation Detection System (Biorad, USA), w buforze 0,5XTBE w 14ºC, 380V przez 21,5 godziny (Grochowska, 2016). Następnie żele zostały wysrebrzone (Silver Stain kit firmy Kucharczyk Techniki Elektroforetyczne Sp. z o.o., Polska), a rezultaty zinterpretowane. Próbki reprezentujące każdy z uzyskanych wzorców SSCP wybrano do sekwencjonowania, które wykonano w firmie Genomed S.A., Polska.

Dane dotyczące cech użytkowości mięsnej uzyskane dla owiec kamienieckich i pomorskich zostały zebrane z dokumentacji hodowlanej

udostępnionej przez Związek Hodowców Owiec i Kóz. Bazy danych dotyczące poszczególnych mas ciała owiec kamienieckich i pomorskich utworzone zostały przez pracowników Katedry Hodowli Owiec i Kóz Wydziału Bioinżynierii Zwierząt Uniwersytetu Warmińsko-Mazurskiego w Olsztynie. Dane liczbowe zebrano dla następujących cech: masy ciała jagniąt w drugim dniu życia (BW2), masa ciała w pięćdziesiątym szóstym dniu życia (BW56), uwzględniając płeć i typ urodzenia jagnięcia. Na podstawie tych danych obliczono średnie dobowe przyrosty masy pomiędzy drugim a pięćdziesiątym szóstym dniem życia (ADG).

Dane dotyczące merynosa barwnego zostały udostępnione przez Zakład Doświadczalny Instytutu Zootechniki PIB w Kołudzie Wielkiej w ramach realizowanego w Zakładzie Genetyki i Podstaw Hodowli Zwierząt Uniwersytetu Technologiczno-Przyrodniczego w Bydgoszczy grantu (N N311 521440) finansowanego ze środków Narodowego Centrum Nauki. Dane dotyczyły masy ciała w 2. dniu życia (BW2), ponadto na podstawie dostępnych danych oszacowano masę ciała w 30. (BW30), 56. (BW56), 70. dniu życia (BW70) oraz średnie dobowe przyrosty masy ciała pomiędzy 2. a 56. dniem (ADG). W przypadku merynosa barwnego, w ramach wyżej wymienionego grantu, dokonano uboju jagniąt oraz podziału tuszy na wyręby, a następnie dysekcji udźca. Określono następujące cechy:

1) masa partii przodu [g],
2) masa partii środka [g],
3) masa partii zadu [g],
4) masa szyi [g],
5) masa goleni przedniej [g],
6) masa karkówki [g],
7) masa łopatki [g],
8) masa łaty z mostkiem [g],
9) masa antrykotu [g],
10) masa combra [g],
11) masa polędwiczki [g],
12) masa goleni tylnej [g],
13) masa udźca [g],
14) masa udźca przed dysekcją [g],
15) zawartość mięśni [g],
16) zawartość tłuszczu [g]
17) zawartość kości w udźcu [g]
18) procentowa zawartość tkanki mięśniowej w udźcu [%],
19) procentowa zawartość tkanki tłuszczowej w udźcu [%],
20) procentowa zawartość tkanki kostnej w udźcu [%].

Analiza statystyczna zgromadzonych danych obejmowała określenie frekwencji genotypów i alleli zidentyfikowanych metodami RFLP i SSCP. Do weryfikacji hipotezy dotyczącej zróżnicowania frekwencji genotypów pomiędzy rasami użyto testu chi^2 (SAS Inc., 2015). Ponadto sprawdzono czy badane populacje znajdowały się w stanie równowagi genetycznej w zakresie genotypów c.*1232G>A. Zgodność rozkładu genotypów z prawem Hardy'ego-Weinberg'a przeprowadzono wykorzystując program Arlequin ver. 3.0 (Excoffier i in., 2005).

Dostępne dane liczbowe dotyczące badanych cech ilościowych scharakteryzowano statystycznie obliczając podstawowe miary położenia i zmienności: średnia (Mean), odchylenie standardowe (Std) oraz współczynnik zmienności (CV). Wpływ badanych czynników na cechy użytkowości mięsnej oceniono przy użyciu wieloczynnikowej analizy wariancji. Analizę wariancji badanych cech wykonano w dwóch wariantach: dla każdej z ras z osobna (Model 1 – model trójczynnikowy) oraz łącznie dla całej badanej populacji (Model 2 – model czteroczynnikowy). W pierwszym modelu liniowym analizę przeprowadzono osobno dla każdej z badanych ras owiec (SAS Inc., 2015).

Model 1.

$$Y_{ijkl} = \mu + a_i + b_j + c_k + (ab)_{ij} + (ac)_{ik} + (bc)_{jk} + e_{ijkl}$$

gdzie:

Y_{ijkl} – badana cecha

μ – średnia

a_i – wpływ płci (samiec, samica)

b_j – wpływ typu urodzenia (jedynak, bliźniak)

c_k – wpływ genotypów określonych metodą RFLP lub SSCP

$(ab)_{ij}$, $(ac)_{ik}$, $(bc)_{jk}$ – interakcje pierwszego stopnia między czynnikami

e_{ijkl} – błąd losowy

Dodatkowo poszerzono analizę statystyczną uwzględniając czynnik rasy. Trzy badane populacje owiec złączono w wykonując analizę statystyczną według następującego modelu.

Model 2.

$$Y_{ijklm} = \mu + a_i + b_j + c_k + d_l + (ab)_{ij} + \ldots + (cd)_{kl} + e_{ijklm}$$

gdzie:

Y_{ijklm} – badana cecha

μ – średnia

a_i – efekt płci (samiec, samica)

b_j – wpływ typu urodzenia (jedynak, bliźniak)

c_k – wpływ genotypów określonych metodą RFLP lub SSCP

d_l – wpływ rasy (kamieniecka, pomorska, merynos barwny)

$(ab)_{ij}+\ldots+(cd)_{kl}$ – interakcje pierwszego stopnia między czynnikami,

e_{ijklm} – błąd losowy

W przypadku cech poubojowych, które określono jedynie w grupie tryczków merynosa barwnego zastosowano uproszczony model liniowy uwzględniający dwa czynniki: typ urodzenia jagnięcia oraz genotyp określony metodą SSCP.

Istotność różnic między grupami płci, typu urodzenia, genotypu i rasy badano za pomocą analizy wariancji i testu Duncan'a. Analizę statystyczną wykonano z zastosowaniem programu SAS (SAS Inc., 2015).

Podczas opracowania statystycznego wyeliminowano grupy genotypów o niewielkiej liczebności. Jeśli grupa nie składała się z przynajmniej pięciu osobników nie była brana pod uwagę podczas dalszego opracowania statystycznego.

9.4. Wyniki

Badania wykazują, że wyekstrahowane DNA było dobrej jakości, o czym świadczyły rezultaty elektroforetycznego rozdziału oraz badania spektrofotometrycznego. Podczas rozdziału na żelu agarozowym otrzymano pojedyncze wyraźne prążki, a podczas badania spektrofotometrem stwierdzono, że stosunek absorpcji A260 do A280 wahał się pomiędzy 1,7 a 1,9, co świadczyło o czystości uzyskanego DNA. Wyniki pomiarów posłużyły do przygotowania rozcieńczeń (50 ng/µl). Analizując produkty uzyskane w trakcie MioClop PCR oraz Hickford PCR, wykazano obecność fragmentów DNA o odpowiedniej długości (1003 pz oraz 414 pz). Wyniki potwierdziły dokładność i specyficzność przeprowadzonych reakcji PCR. Produkty pozyskane podczas MioClop PCR zostały wykorzystane podczas reakcji RFLP, a Hickford PCR podczas SSCP.

Określono strukturę genetyczną badanych populacji pod względem frekwencji genotypów oznaczonych techniką RFLP i SSCP. W przypadku analizy polimorfizmu c.*1232G>A z wykorzystaniem enzymu restrykcyjnego *Hpy*CH4IV (NEB, USA) zidentyfikowano 2 allele (A i G) oraz 3 genotypy

(AA, AG, GG). Zdjęcie 1. przedstawia obraz żelu agarozowego z uzyskanymi wzorcami prążków odpowiadającymi genotypom wykrytym metodą RFLP, a tabela 9 frekwencje genotypów pod względem c.*1232. W populacji owiec kamienieckich i pomorskich najrzadziej występował genotyp AA (jedynie 6%). Częstość wystąpienia genotypu AG różniła się w zależności od rasy. Najczęściej genotyp ten wykrywano u owiec pomorskich (56%), zdecydowanie rzadziej u kamienieckich (21%) (Tabela 9). W populacji merynosa barwnego stwierdzono występowanie jedynie allela G (Tabela 10). W pozostałych rasach wykryto również allel A. Frekwencja allela A była najwyższa w przypadku grupy owiec pomorskich (34%). W populacji owiec kamienieckich frekwencja ta wynosiła 16,5%. 83,5% owiec kamienieckich oraz 66% owiec pomorskich było nosicielami allela G (Tabela 10).

W przypadku grupy owiec kamienieckich i pomorskich sprawdzono czy populacje znajdowały się w równowadze Hardy-Weinberg'a. Obserwowany udział heterozygot AG w populacji owiec kamienieckich i pomorskich wynosił odpowiednio: 0,21 oraz 0,56, zaś heterozygotyczność oczekiwana wynosiła: 0,28 i 0,45. Wartości P (0,024 dla owiec kamienieckich i 0,023 pomorskich) obliczone z wykorzystaniem programu Arlequin ver. 3.0 (Excoffier i in., 2005) pozwoliły stwierdzić, że obie populacje nie były w równowadze genetycznej. Populacja merynosa barwnego była monomorficzna pod względem alleli c.*1232, co świadczyło o tym, że również nie znajdowała się w równowadze.

Wyniki reakcji SSCP zostały zaprezentowane na zdjęciu 2. Z kolei frekwencje genotypów przedstawiono w tabeli 11. W celu odróżnienia alleli zidentyfikowanych w niniejszej pracy od tych opisanych przez Hickford'a i in. (2009) zastosowano następujące nazewnictwo: A_1-F_1. Frekwencje poszczególnych genotypów wykrytych metodą SSCP były zróżnicowane pomiędzy rasami. Jedynie trzy genotypy wykryte metodą SSCP były obecne we wszystkich trzech badanych rasach: A_1B_1, A_1A_1 oraz A_1C_1. Ich frekwencje znacznie się różniły w zależności od rasy. W populacji owiec kamienieckich najczęściej występującym genotypem był genotyp A_1B_1 (51%), u owiec pomorskich i merynosa barwnego najczęściej występował genotyp A_1A_1 (Tabela 11). Najczęściej występującym allelem był allel A_1 (54,1% u owiec kamienieckich, 90% u merynosa barwnego i 95% u owiec pomorskich). Częstość występowania innych alleli różniła się w poszczególnych rasach (Tabela 12).

Genotypy zidentyfikowane dzięki zastosowaniu techniki SSCP zsekwencjonowano. Sekwencje uzyskane dla każdego wzorca zostały porównane z genomem referencyjnym (NCBI Reference Sequence: NC_019459.1, *Ovis aries* breed Texel chromosome 2, Oar_v3.1) poprzez wykorzystanie opcji BLAST (BLAST, 2015). Porównanie to wykazało, że sekwencja genotypu A_1A_1 była w 100% zgodna z sekwencją referencyjną.

Sekwencje porównano również za pomocą programu MUSCLE 3.8 (Edgar, 2004a,b), a wyniki przedstawiono na schemacie 3. Zidentyfikowano osiem miejsc polimorficznych w pozycji następujących nukleotydów (poczynając od pierwszego nukleotydu w genie miostatyny): 391., 474., 614., 616., 619., 622., 632. i 696. Różnice w sekwencjach pomiędzy poszczególnymi allelami oraz genotypami zostały przedstawione w tabelach 13. i 14.

Dostępne dane liczbowe zostały opracowane statystycznie pod względem badanych cech użytkowości mięsnej, uwzględniając czynnik genotypu oraz rasy, a wyniki przedstawiono w tabelach 15-18. Analiza statystyczna wykorzystująca liniowy model 1 nie potwierdziła statystycznie istotnego wpływu genotypów, wykrytych przy użyciu RFLP oraz SSCP na cechy użytkowości mięsnej.

Podczas opracowania statystycznego mającego na celu ustalenie wpływu polimorfizmu miostatyny na cechy poubojowe wykorzystano jedynie populację merynosa barwnego, która była monomorficzna pod względem mutacji c.*1232G>A oraz składała się z samych tryczków. Jedynie genotypy wykryte dzięki technice SSCP oraz typ urodzenia były brane pod uwagę podczas opracowania statystycznego. Analiza nie wykazała wpływu tych genotypów na cechy poubojowe (Tabela 20a i 20b). Mogło mieć to związek z faktem, że badano niewielką grupę zwierząt o nierównej liczebności porównywanych grup.

Dodatkowo podczas opracowania statystycznego przeprowadzono analizę wariancji z wykorzystaniem poszerzonego modelu liniowego (Model 2), do którego dodano czynnik rasy. Analiza statystyczna wpływu poszczególnych genotypów c.*1232 zidentyfikowanych za pomocą techniki RFLP na masy ciała (BW2, BW56, ADG) wykazała, że zwierzęta posiadające genotyp AG charakteryzowały się najwyższą masą ciała w 56. dniu życia oraz najwyższymi przyrostami masy ciała (Tabela 21). W przypadku genotypów wykrytych metodą SSCP wykazano, że najwyższe masy ciała w 56. dniu życia i przyrosty dobowe charakteryzowały zwierzęta o genotypie A_1A_1, a najniższe o genotypie B_1B_1 (Tabela 21).

Wyniki analizy wpływu czynników płci i typu urodzenia na masy ciała i cechy poubojowe w przypadku gdy do opracowania statystycznego zastosowano model 1 przedstawiono w tabelach 19 – 20. Tabela 22 przedstawia zaś wpływ trzech badanych czynników (płci, typu urodzenia oraz rasy) na masy ciała jagniąt w przypadku zastosowania modelu 2. Podczas analizy statystycznej stwierdzono statystycznie istotny wpływ płci, typu urodzenia oraz rasy na poszczególne masy ciała oraz na niektóre cechy poubojowe.

9.5. Dyskusja

Szybki rozwój technik genetyki molekularnej może ułatwić ocenę zwierząt oraz prowadzenie ukierunkowanej selekcji. Możliwe staje się stosowanie testów molekularnych w celu identyfikacji osobników posiadających pożądany genotyp.

W badaniach własnych w celu identyfikacji genotypów pod względem genu *GDF*8 zastosowano trzy techniki molekularne, a mianowicie techniki PCR-RFLP, PCR-SSCP oraz sekwencjonowanie. Ta pierwsza zastosowana została do identyfikacji mutacji c.*1232G>A, druga natomiast do ustalenia polimorfizmów w obrębie fragmentu intronu pierwszego o długości 414 par zasad. Ostatnia z wymienionych technik posłużyła do poznania sekwencji nukleotydowej osobników posiadających poszczególne genotypy wykryte metodą SSCP. Różne techniki były stosowane również przez innych autorów. Technika PCR-RFLP była wykorzystywana między innymi przez Clop i in. (2006), Johnson i in. (2009), Jamshidi i in. (2014), Akbari i in. (2015) czy Georgieva i in. (2015). Z kolei metodę SSCP zastosowali między innymi następujący badacze: Zhou i in. (2008), Hickford i in. (2009), Han i in. (2010), Ansary i in. (2011), Farhadian i in. (2011), Sumantri i in. (2011) etc.

Wielu autorów badało intron pierwszy genu miostatyny za pomocą metody SSCP, jednakże uzyskane przez nich obrazy prążków znacznie się różniły. Miało to związek z kilkoma czynnikami. Po pierwsze nie wszyscy autorzy amplifikowali ten sam fragment genu. Fragment o długości 414 pz, taki sam jak w niniejszych badaniach, wykorzystali m.in. Hickford i in. (2009) oraz Farhadian i in. (2012). Jednakże w obu przypadkach parametry elektroforezy znacząco się różniły od tych zastosowanych w niniejszej pracy. W badaniach własnych próbki zostały naniesione na 8,5% żel poliakrylamidowy, a elektroforeza przeprowadzona została w następujących warunkach: 380V, 14ºC przez 21,5 godziny (Grochowska, 2016). Wymienione parametry różniły się od tych zastosowanych przez Hickforda i in. (2009), którzy zastosowali 12% żel, a elektroforezę przeprowadzili w następujących warunkach: 12ºC, 18 godzin, 350V. Farhadian i in. (2012) z kolei wykorzystali 8% żel poliakrylamidowy i następujące parametry: 18ºC, 200V, 3 godziny. W obu przypadkach zmiany powodowały powstanie odmiennych wzorców SSCP. Inni autorzy opisywali badania różnych części pierwszego intronu genu miostatyny. Zhou i in. (2008) zamplifikowali partię genu składającą się z części 5'UTR, pierwszej kodującej domeny oraz początku intronu pierwszego. Zastosowali również odmienne warunki samej elektroforezy. Rozdział w 14% żelu poliakrylamidowym, przy 400V i w 4ºC trwał u nich 18 godzin. Ansary i in. (2011) w swoich badaniach amplifikowali fragment pierwszego intronu genu *GDF*8 o długości 291 pz. Rozdział SSCP przeprowadzili w 8% żelu

poliakrylamidowym w 6ºC, przy 250V przez 8 godzin. Z kolei Dehnavi i in. (2012) do swoich badań wybrali fragment intronu pierwszego o długości 222 pz i rozdzielili go na 10% żelu poliakrylamidowym, w temperaturze 10ºC, przy 250V, a czas trwania elektroforezy wynosił 4 godziny.

Kolejną techniką zastosowaną podczas badań własnych było sekwencjonowanie. Metoda ta została wykorzystana również przez Boman i Våge (2009). Oprócz wymienionych już narzędzi genetyki molekularnej, autorzy stosowali inne techniki, na przykład Boman i in. (2011) zastosowali platformę SEQUENOM MassARRAY, Haynes i in. (2012) technikę real-time PCR, a Masri i in. (2011a) wykorzystali komercyjnie dostępny test MyoMAX®, który identyfikuje mutację c.*1232G>A.

Uzyskane wyniki wskazują, że występowanie polimorfizmu c.*1232G>A w obrębie genu różni się w zależności od badanej rasy. Wyniki te są zgodne z informacjami publikowanymi w literaturze światowej. Clop i in. (2006), Kijas i in. (2007) oraz Hadjipavlou i in. (2008) stwierdzili, iż w obrębie rasy texel frekwencja allela A wynosi 99%; Bignell i in. (2009) wykazali, że frekwencja tego allela w populacji owiec wschodnio – fryzyjskich była na poziomie 63%. Z kolei w grupie owiec merynosa dzhalginsky 80% osobników posiadało allel A (Trukhachev i in., 2015). Na niższym poziome frekwencje allela A u owiec texel × welsh mountain zanotowali Masri i in. (2011a). Allel A posiadało niecałe 54% osobników. Hadjipavlou i in. (2008) donosili, że allel c.*1232A występował u 30% osobników owcy charollais, a Han i in. (2010), że występował u 22,8% owiec romney. Zdecydowanie niższe frekwencje allela A odnotowane zostały przez Han i in. (2015) (2.79% u owiec romney), Kijas i in. (2007) (12.5% u owiec lincoln, poll dorset i white suffolk). Inni autorzy w ogóle nie wykryli obecności allela c.*1232A (Hadjipavlou i in., 2008; Miar i in., 2011).

W badaniach własnych allel c.*1232A został wykryty jedynie u osobników rasy kamienieckiej i pomorskiej. Badana populacja owiec pomorskich była bardziej zróżnicowana pod względem alleli c.*1232 i owce tej rasy częściej były nosicielami dwóch różnych alleli niż owce kamienieckie. Obecność allela A w populacji owiec kamienieckich i pomorskich może wynikać z faktu, iż w przeszłości w celu poprawienia użytkowości mięsnej krzyżowano maciorki tych ras z trykami rasy texel (Kawęcka i Sikora, 2012). Jak wskazuje Boman i in. (2011) w wyniku intensywnych prac hodowlanych można zwiększyć frekwencje danego allela.

W niniejszych badaniach fragment intronu 1 genu miostatyny o długości 414 par zasad został zamplifikowany i poddany rozdziałowi SSCP. Stwierdzono wystąpienie ośmiu różnych genotypów i 6 alleli (A_1-F_1). Jedynie trzy genotypy zidentyfikowano we wszystkich trzech badanych rasach (A_1B_1, A_1A_1 oraz A_1C_1), a ich frekwencje znacząco się różniły w każdej z badanych grup.

W grupie owiec kamienieckich najczęściej występował genotyp A_1B_1 (51%), a następnie A_1A_1 (25%) oraz B_1B_1 (20%). Frekwencje pozostałych genotypów nie przekraczały poziomu 2%. W populacji merynosów barwnych stwierdzono, że najwięcej osobników posiadało genotyp A_1A_1 (79,4%). W populacji owiec pomorskich aż 89% osobników posiadało dwa allele A_1. We wszystkich trzech populacjach najczęściej występował allel A_1 (51,5%, 89,69% oraz 94,5% odpowiednio w grupie owiec kamienieckich, merynosa oraz owiec pomorskich). Allel B_1 najczęściej występował u owiec kamienieckich (46,5%). W dwóch pozostałych badanych rasach jego frekwencja pozostawała na bardzo niskim poziomie. Badania mające na celu poznanie frekwencji poszczególnych alleli genu *GDF*8 przeprowadzone zostały również przez innych badaczy (np. Hickford i in., 2009; Farhadian i in., 2012). Hickford i in. (2009) badali fragment intronu pierwszego genu miostatyny. Zidentyfikowali oni pięć alleli i oznaczyli je literami od A do E. W nowozelandzkiej populacji owiec romney zidentyfikował jedynie trzy allele (A, B i C), które składały się na 6 genotypów. Frekwencje tych genotypów były następujące: AA (46,6%), AB (30,2%), AC (13,3%), BB (5,8%), BC (3,5%) oraz CC (0,6%). Najczęściej występował allel A (68,3%), a najrzadziej allel C (9%). Farhadian i in. (2012) zidentyfikowali cztery genotypy SSCP w obrębie pierwszego intronu: AD, AC, AE oraz BC (odpowiednio: 41,3%, 29,3%, 13% oraz 16,3%). Najwyższą frekwencją charakteryzował się allel A (41,85%). Frekwencje alleli C i D były znacznie niższe (22,83% oraz 20,65%). Inni badacze również badali polimorfizm w pierwszym intronie genu miostatyny, jednakże zidentyfikowane wzorce nazywali według innej nomenklatury. Jednym z przykładów może być praca Zhou i in. (2008), którzy zidentyfikowali genotypy *01 (86%), *02 (12%) i *03 (2%). Z kolei Han i in. (2015) opisali wystąpienie u tryków romney pięciu alleli: H1 (2,79%), H2 (53,13%), H4 (24,55%), H5 (15,19%) i H7 (4,34%). Wykazali oni, że najczęściej występującymi genotypami w badanej populacji były genotypy H2H2 (34,08%), H3H2 (26,78%) oraz H5H2 (16,34%). Inne nazwy haplotypów SSCP (P1, P2 i P3) zaproponowali Ansary i in. (2011). Badali oni polimorfizm w 291 nukleotydowym fragmencie intronu pierwszego genu *GDF*8 w populacji irańskich owiec baluchi.

Ponieważ zarówno badany region intronu pierwszego genu miostatyny jak i parametry rozdziału SSCP znacznie się różniły od tych opisanych w publikacjach innych autorów, konieczne było sekwencjonowanie próbek dostępnych dla każdego genotypu wykrytego podczas niniejszych badań własnych. Ułatwiło to porównane wyników z tymi uzyskanymi przez innych badaczy. W niniejszych badaniach wykryto osiem genotypów, a próbki reprezentujące każdy z nich zsekwencjonowano. Otrzymane sekwencje porównano z genomem referencyjnym (Oar_v3.1). Porównanie to wykryło osiem SNP: g.391G>T, g.474C>T; g.614T>C; g.616G>A; g.619T>C; g.622T>C; g.632G>T; g.696C>T. Genotypy te nie były obecne w każdej

z badanych ras. Wyniki uzyskane w niniejszych badaniach wykazały obecność mutacji wcześniej już opisanych w literaturze. Jedni z pierwszych badaczy którzy opisali niektóre z tych mutacji byli Clop i in. (2006) badający SNP w obrębie genu miostatyny owiec texel. Ich zespół w intronie pierwszym zidentyfikował 7 SNP, które odpowiadały tym zidentyfikowanym w niniejszych badaniach własnych. Clop i in. (2006) nie wykryli jedynie obecności mutacji g.614T>C. Tę mutację, razem z 7 innymi, opisał za to Hickford i in. (2009) u owiec nowozelandzkiej rasy romney. Zidentyfikowali oni 5 alleli (A-E) oraz ustalili sekwencję nukleotydową dla każdego z miejsc polimorficznych. Należy zaznaczyć, że sekwencje te były podobne do tych uzyskanych w niniejszych badaniach. Jedynym wyjątkiem była mutacja g.474C>T. Podczas gdy Hickford i in. (2009) stwierdzili, że w pozycji 474. nukleotydu (począwszy od początku genu) znajduje się tymina, wyniki niniejszych badań własnych wskazują, że w allelach od A_1 do E_1 w pozycji 474. nukleotydu znajduje się cysteina. Jedynie sekwencja allela F_1 była identyczna jak ta opisywana przez Hickforda i in. (2009) dla allela E (Tabela 13). Han i in. (2013) przeprowadzili badanie na zróżnicowanej rasowo grupie owiec (owce romney, coopworth, corriedale, dorper, perendale, suffolk, merynos, dorset down, poll dorset, texel oraz owce pochodzące z krzyżowania z owcami nowozelandzkimi) w celu ustalenia czy polimorfizm w jego obszarze zależny jest od rasy. W obszarze intronu pierwszego opisali oni 11 SNP, z czego 7 pokrywało się z tymi opisanymi w niniejszej pracy. Han i in. (2013) w żadnej z badanych populacji nie wykryli mutacji g.474C>T. Zauważyli oni również silne zróżnicowanie pod względem występowania poszczególnych mutacji pomiędzy rasami. Kolejnym zespołem, który badał sekwencję pierwszego intronu genu miostatyny był zespół Sjakste i in. (2011). Badali oni próbki pobrane od łotewskiej owcy czarnogłówki. Zidentyfikowali oni 4 SNP opisane w niniejszych badaniach własnych: c.373+18T>G (391. nt), c.373+241C>T (614. nt), c.373+243A>G (616. nt), c.373+259T>G (632. nt) oraz 2 SNP nieopisane w obecnej dysertacji: c.373+434N>A, c.373+435C>A. Z kolei Trukhachev i in. (2015) badali rosyjskie owce merynosa dzhalginsky. W obrębie intronu pierwszego zidentyfikowali następujące mutacje: c.373+18, c.373+241, c.373+243, c.373+259, c.373+563. Trukhachev i jego zespół nie potwierdzili wystąpienia trzech mutacji (g.474C>T, g.622T>C i g.696C>T). Wszystkie powyższe wyniki wskazują na to, że wystąpienie poszczególnych mutacji w obrębie pierwszego intronu genu *GDF*8 różni się w zależności od rasy.

Jednym z głównych celów niniejszych badań było zbadanie powiązania polimorfizmu c.*1232G>A z cechami użyteczności mięsnej trzech polskich ras owiec. W trzech badanych populacjach stosując Model 1 nie udało się stwierdzić statystycznych zależności, jednakże gdy grupy połączono w jedną populację i powtórzono analizę (Model 2) stwierdzono, że genotyp w pozycji c.*1232 ma statystycznie istotny wpływ na masę ciała w 2. dniu życia (BW2),

w 56. dniu (BW56) oraz na przyrosty dobowe (ADG). Najwyższe wartości BW2 stwierdzono w odniesieniu do osobników homozygotycznych pod względem allela G. Wyniki te pozostają w sprzeczności do tych opublikowanych przez innych autorów, którzy allel A uważają za bardziej pożądany pod względem masy ciała (Han i in., 2010; Haynes i in., 2013; Han i in., 2015). Istnieją również badania nie wykazujące wpływu allela A na cechy mięsności owiec texel i charollais (Hadjipavlou i in., 2008; Johnson i in., 2009).

Najwyższą masę ciała w 56. dniu życia oraz najwyższe przyrosty dobowe w badaniach własnych osiągnęły owce o genotypie AG (c.*1232AG), podczas gdy najniższą - osobniki o genotypie GG (c.*1232GG). Inni autorzy otrzymali podobne wyniki. Han i in. (2015) wykazali, że owce będące nosicielami allela A charakteryzowały się zwiększoną masą poszczególnych partii tuszy. Kolejnymi autorami, którzy powiązali wystąpienie allela A ze zwiększeniem masy ciała, byli między innymi Hadjipavlou i in. (2008), Masri i in. (2011a, 2011b) oraz Haynes i in. (2013). Hope i in. (2013) wskazali, że wystąpienie genotypu AA może być powiązane ze zwiększeniem masy poszczególnych wyrębów oraz zmniejszeniem udziału tkanki tłuszczowej w tuszy. Z kolei Trukhachev i in. (2015) podał, że owce należące do rasy merynosa dzhalginsky charakteryzowały się większą nawet o 2,2% długością tuszy.

W przypadku analizy wpływu genotypów wykrytych metodą SSCP na masy ciała jagniąt najwyższe wartości masy ciała w drugim dniu życia stwierdzono u osobników posiadających genotyp A_1E_1. Zaobserwowane różnice były statystycznie istotne. Najwyższe wartości dla masy ciała w 56. dniu życia i przyrostów dobowych pomiędzy 2. a 56. dniem życia charakteryzowały jagnięta posiadające genotyp A_1A_1, a najniższe – osobniki homozygotyczne pod względem allela B_1. Nie stwierdzono natomiast statystycznie istotnego wpływu genotypów na cechy poubojowe, co mogło mieć związek z faktem, że badana grupa była niewielka i składała się jedynie z jagniąt jednej płci. Wpływ polimorfizmu w obrębie intronu pierwszego badali również inni autorzy. Hickford i in. (2009) powiązali wystąpienie allela A z pogorszeniem się wydajności rzeźnej, a allela B ze zwiększeniem masy polędwicy. Badacze ci zauważyli również, że osobniki posiadające dwa allele B charakteryzowały się zmniejszoną masą łopatki. Ponadto wykazali oni oddziaływanie genotypów wykrytych metodą SSCP na masy ciała przy urodzeniu. Farhadian i in. (2012) również przeprowadzali badania nad wpływem SNP w obrębie intronu 1 na różne cechy związane z masą ciała. W irańskiej populacji owiec makoei stwierdzili oni, że spośród czterech wykrytych genotypów jedynie jeden (AD) istotnie wpływał na urodzeniową masę ciała. Ansary i in. (2011) zidentyfikowali trzy genotypy w intronie 1 i powiązali wystąpienie genotypu P1 ze zwiększonymi masami ciała przy urodzeniu, odstawieniu oraz w wieku sześciu miesięcy. Trukhachev i in. (2015) stwierdzili, że zwierzęta będące homozygotami pod względem mutacji c.373+18G>T (g.391G>T) znacząco się

różniły od osobników nie posiadających tej mutacji w odniesieniu do długości zadu i szerokości combra.

W badaniach własnych dla wszystkich trzech ras wykazano istotny wpływ typu urodzenia na masę ciała w poszczególnych dniach życia. Jedynaki uzyskiwały wyższe masy niż owce urodzone w miocie. Wyniki te są zgodne z doniesieniami publikowanymi w literaturze światowej (Gardner i in., 2007; Chniter i in., 2011; Simeonova i in., 2014). W badaniach własnych wykazano wpływ typu urodzenia na masy poszczególnych wyrębów. Jedynaki osiągały wyższe wartości masy dla antrykotu oraz składu tkankowego udźca. Jest to zgodne z wynikami uzyskanymi przez Ekiz i in. (2012), którzy również stwierdzili, iż masa poszczególnych wyrębów była wyższa w przypadku jagniąt urodzonych w pojedynkę. Z kolei Simeonova i in. (2014) nie znaleźli powiązania pomiędzy typem urodzenia a cechami tusz jagnięcych.

Ponadto, w niniejszych badaniach wykazano wpływ płci na masę jagniąt. Samce charakteryzowały się większą masą ciała w stosunku do samic, jednakże nie stwierdzono istotnego wpływu płci na dzienne przyrosty masy ciała. Rezultaty uzyskane w badaniach własnych są zgodne z uzyskanymi przez Gardnera i in. (2007). Stwierdzili oni, iż samce osiągały wyższe wartości mas ciała niż samice. Z kolei Daskiran i in. (2010) oraz Simeonova i in. (2014) stwierdzili powiązanie pomiędzy płcią jagnięcia a średnimi przyrostami masy ciała. Zauważyli oni, że samce charakteryzowały się większymi przyrostami dobowymi. Te dane stoją w sprzeczności z doniesieniem Manso i in. (1996), którzy stwierdzili, iż pomimo, że samice urodziły się z niższą masą ciała niż samce, osiągały one wyższe wartości przerostów dobowych.

9.6. Podsumowanie i wnioski

Na podstawie wyników przeprowadzonych badań można stwierdzić, że:

- Wyizolowane DNA było dobrej jakości, czego dowiódł między innymi rozdział elektroforetyczny oraz pomiar spektrofotometryczny. Zastosowane metody badawcze pozwoliły zidentyfikować polimorfizm w obrębie genu *GDF*8 w pozycji c.*1232 oraz w badanym fragmencie pierwszego intronu. Potwierdzono wystąpienie mutacji we fragmencie pierwszego intronu za pomocą sekwencjonowania.
- Stosując metodę RFLP w obrębie genu *GDF*8, w populacji owcy kamienieckiej i pomorskiej, zidentyfikowano dwa allele (A i G) i trzy genotypy (AA, AG i GG), natomiast w populacji merynosa barwnego zidentyfikowano jedynie allel G. Frekwencje alleli i genotypów w poszczególnych rasach znacznie się różniły. Najwyższą frekwencją w populacji owcy kamienieckiej i merynosa barwnego charakteryzował się

genotyp GG, podczas gdy w populacji owcy pomorskiej najczęściej występował genotyp AG. W odniesieniu do polimorfizmu c.*1232G>A badane populacje nie znajdowały się w równowadze genetycznej.

- W obrębie pierwszego intronu genu *GDF*8 zidentyfikowano 8 genotypów (A_1A_1, A_1B_1, A_1C_1, A_1D_1, A_1E_1, A_1F_1, B_1B_1, B_1C_1) oraz 6 alleli (A_1-F_1). Zarówno obecność poszczególnych mutacji jak i ich frekwencje różniły się pomiędzy rasami. Najwyższą frekwencję w grupie owiec kamienieckich odnotowano dla genotypu A_1B_1, podczas gdy w pozostałych badanych rasach jego frekwencja była bardzo niska. W populacji owcy pomorskiej i merynosa barwnego najczęściej występował genotyp B_1B_1. Wystąpienie genotypu A_1F_1 odnotowano jedynie w populacji merynosa barwnego.
- Zsekwencjonowanie fragmentu intronu pierwszego o długości 414 nukleotydów potwierdziło obecność ogółem ośmiu miejsc polimorficznych: g.391G>T, g.474C>T; g.614T>C; g.616G>A; g.619T>C; g.622T>C; g.632G>T; g.696C>T. Odnotowano niewielkie różnice pomiędzy rasami – mutacja g.474C>T wystąpiła jedynie w populacji jagniąt merynosa barwnego, a mutacja g.619T>C wyłącznie w populacji owcy pomorskiej i kamienieckiej.
- Analizując powiązanie polimorfizmów w obrębie genu *GDF*8, określonych metodami RFLP i SSCP z masami ciała jagniąt, w obrębie każdej rasy osobno, nie stwierdzono statystycznie istotnego zróżnicowania tych cech w zależności od badanych genotypów. Po włączeniu do analizy wariancji czynnika rasy wykazano statystycznie istotny wpływ tych polimorfizmów na masy ciała jagniąt w 2. i 56. dniu życia oraz na przyrosty dobowe pomiędzy 2. a 56. dniem życia. W przypadku genotypów określonych metodą RFLP, najwyższą masą ciała w 56. dniu życia oraz najwyższymi dziennymi przyrostami masy ciała pomiędzy 2. a 56. dniem życia odznaczały się jagnięta o genotypie AG. Dla genotypów zidentyfikowanych metodą SSCP, najwyższą masę ciała w 56. dniu życia wykazywały osobniki o genotypie A_1A_1.
- W zakresie badanych cech poubojowych nie stwierdzono statystycznie istotnego zróżnicowania między genotypami określonymi techniką SSCP.
- W celu pełniejszego wyjaśnienia powiązania polimorfizmów w obrębie genu *GDF*8 z cechami użytkowości mięsnej wskazane byłoby objąć badaniami większą i bardziej zróżnicowaną rasowo populację.

10. REFERENCES

[1] Akbari S., Dashab G.R., Alipanah M., Rokouei M., Saghi D.A., 2015. Study on the polymorphism in myostatin gene and its association with growth traits in Kordi sheep of Northern Khorasan. Modern Genetics Journal (MGJ) 10(1), 129-133.

[2] Allais S., Levéziel H., Payet-Duprat N., Hocquette J. F., Lepetit J., Rousset S., Denoyelle C., Bernard-Capel C., Journaux L., Bonnot A., Renand G., 2010. The two mutations, Q204X and nt821, of the myostatin gene affect carcass and meat quality in young heterozygous bulls of French beef breeds. Journal of Animal Science 88, 446-454.

[3] Ansary M., Tahmoorespur M., Nassiry M.R., Taheri A., Vafaye Valeh M., 2011. Polymorphism in Intron-I of Myostatin gene and its association with estimated breeding values of growth traits in Baluchi sheep (Ovisaries). The Indian Journal of Animal Sciences 81(8), 75-78.

[4] Awole Adem M., 2006. The Polymerase Chain Reaction [in] Awole Adem M. Molecular biology and applied genetics for medical laboratory technician students lecture note series. Jimma University.

[5] Baranowski P., 1998. Wybrane importowane i krajowe rasy oraz odmiany owiec – historia ich powstania. Uczelniane Akademii Rolniczej w Szczecinie p, 20-22.

[6] Bellinge R.H.S., Liberles D.A., Iaschi S.P.A., O'Brien P.A., Tay G.K., 2005. Myostatin and its implications on animal breeding: a review. Animal Genetics 36, 1-6.

[7] Bignell C.W., Malau-Aduli A.E.O., Nichols P.D., McCulloch R., Kijas J.W., 2009. East Friesian sheep carry a myostatin allele known to cause muscle hypertrophy in other breeds. Animal Genetics 41, 445. doi:10.1111/j.1365-2052.2010.02021.x.

[8] BLAST, 2015, www.blast.ncbi.nlm.nih.gov [Retrieved 24 October 2015]

[9] Boman I.A., Klemetsdal G., Blichfeldt T., Nafstad O., Vage D.I., 2009. A frameshift mutation in the coding region of the myostatin gene (MSTN) affects carcass conformation and fatness in Norwegian White Sheep (Ovisaries). Animal Genetics 40, 418-422.

[10] Boman I.A., Klemetsdal G., Nafstad O., Blichfeldt T., Vage D.I., 2011. Selection based on progeny testing induces rapid changes in myostatin allele frequencies – a case study in sheep. Journal of Animal Breeding and Genetics 128, 52-55. doi:10.1111/j.1439-0388.2010.00879.x

[11] Boman I.A., Klemetsdal G., Nafstad O., Blichfeldt T., Våge D.I., 2010. Impact of two myostatin (MSTN) mutations on weight gain and lamb carcass classification in Norwegian White Sheep (Ovis aries). Genetics Selection Evolution 42, 4-11.

[12] Boman I.A., Våge D.I., 2009. An insertion in the coding region of the myostatin (MSTN) gene affects carcass conformation and fatness in the Norwegian Spælsau (Ovis aries). BMC Research Notes 2, 98. doi:10.1186/1756-0500-2-98
[13] Caestecker M., 2004. The transforming growth factor-beta superfamily of receptors. Cytokine & growth factor reviews 15, 1-11.
[14] Charon K.M., Świtoński M., 2006. Genetyka zwierząt. Wydanie naukowe PWN ISBN/ISSN: 83-01-14022-4, p: 78-82, 86-87.
[15] Chniter M., Hammadi M., Khorchani T., Krit R., Lahsoumi B., Sassi M.B., Nowak R., Hamoud M.B., 2011. Phenotypic and seasonal factors influence birth weight, growth rate and lamb mortality in D'man sheep maintained under intensive management in Tunisian oases. Small Ruminant Research 99(2011), 166-170.
[16] Cloete J.J.E., Cloete W.P., Olivier J.J., Hoffman L.C., 2007. Terminal cross-breeding of Dorper ewes to Ile de France, Merino Landsheep and SA, Mutton Merino sires: ewe production and lamb performance. Small Ruminant Research 69, 28-35.
[17] Clop A., Marcq F., Takeda H., Pirottin D., Tordoir X., Bibe B., Bouix J., Caiment F., Elsen J.M., Eychenne F., Larzul C., Laville E., Meish F., Milenkovic D., Tobin J., Charlier C., Georges M., 2006. A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. Nature Genetics 38, 813-818.
[18] Dankowski A., Bernacka H., Janicki B., Siminska E., 2005. Użytkowanie owiec. Wydawnictwo Uczelniane Akademii Techniczno-Rolniczej w Bydgoszczy, Bydgoszcz p, 43-49
[19] Daskiran, I., Koncagul, S., Bingol, M., 2010. Growth characteristics of indigenous Norduz female and male lambs. Journal of Agricultural Science 16, 62-69.
[20] Dehnavi E., Azari M.A., Hasani S., Nassiry M.R., Mohajer M., Ahmadi A.K., Shahmohamadi L., Yousefi S., 2012. Polymorphism of Myostatin Gene in Intron 1 and 2 and Exon 3, and Their Associations with Yearling Weight, Using PCR-RFLP and PCR-SSCP Techniques in Zel Sheep. Biotechnology Research International doi:10.1155/2012/472307 (ID 472307)
[21] Dunner S., Miranda M.E., Amigues Y., Cañón J., Georges M., Hanset R., Williams J., Ménissier F., 2003. Haplotype diversity of the myostatin gene among beef cattle breeds. Genetics Selection Evolution 35, 103-118.
[22] Edgar R.C., 2004a. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32(5), 1792-1797. doi:10.1093/nar/gkh340

[23] Edgar R.C., 2004b. MUSCLE: a multiple sequence alignment method with reduced time and space complexity BMC Bioinformatics 5, 113. doi:10.1186/1471-2105-5-11
[24] Ekiz B., Ekiz E., Yalcintan H., Kocak O., Yilmaz A., 2012. Effects of suckling length (45, 75 and 120 d) and rearing type on cortisol level, carcass and meat quality characteristics in Kivircik lambs. Meat Science 92, 53-61.
[25] Elkasrawy M.N., Hamrick M.W., 2010. Myostatin (*GDF*-8) as a key factor linking muscle mass and bone structure. Journal of Musculoskeletal and Neuronal Interactions 10(1), 56-63.
[26] Elkina Y., Von Haehling S., Anker S. D., Springer J., 2011. The role of myostatin in muscle wasting: an overview. Journal of Cachexia, Sarcopenia and Muscle 2, 143-151.
[27] Ensemble, 2016. http://www.ensembl.org/Ovis_aries/Gene/Summary?db=core;g=ENSOARG00000016285;r=2:118144443-118149433;t=ENSOART00000017734 [Retrieved 5 January 2016]
[28] Excoffier, L. G. Laval, and S. Schneider, 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1, 47-50.
[29] Farhadian M., Hashemi A., Mardani K., Darvishzadeh R., Jafari S., 2012. Polymorphisms in the ovine myostatin gene are associated with birth weight but not with weight gain in Iranian Makoei sheep. Genetics and Molecular Research 11(4), 3568-3575.
[30] Farhadian M., Hashemi A., Mardani K., Darvishzadeh R., Ranjbari M., 2011. Allelic polymorphism of 'Makoei' sheep myostatin gene identified by polymerase chain reaction and single strand conformation polymorphism. African Journal of Biotechnology 10(50), 10083-10086.
[31] Gardner D.S., Buttery P.J., Daniel Z., Symonds M.E., 2007. Factors affecting birth weight in sheep: maternal environment. Reproduction 133(1), 297-307.
[32] Georges M., 2010. When Less Means More: Impact of Myostatin in Animal Breeding. Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry 10, 240-248.
[33] Georgieva S., Hristova D., Dimitrova I., Stancheva N., Bozhilova-Sakova M., 2015. Molecular analysis of ovine calpastatin (CAST) and myostatin (MSTN) genes in Synthetic Population Bulgarian Milk sheep using PCR-RFLP. Journal of Bioscience and Biotechnology 4(1), 95-99.
[34] Grobet L., Poncelet D., Royo L.J., *et al.*, 1998. Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. Mammalian Genome 9, 210-213.
[35] Grochowska, 2016 (unpublished manuscript)

[36] Guerrero A., Velandia Valero M., Campo M.M., Sañudo C., 2013. Some factors that affect ruminant meat quality: from the farm to the fork. Review. Acta Scientiarum. Animal Sciences 35(4), 335-347.
[37] GUS, 2014. Główny Urząd Statystyczny. Departament Rolnictwa., 2014. Pogłowie bydła i owiec według stanu w czerwcu 2014 r. http://stat.gov.pl/obszary-tematyczne/rolnictwo-lesnictwo/produkcja-zwierzeca-zwierzeta-gospodarskie/poglowie-bydla-i-owiec-wedlug-stanu-w-czerwcu-2014-r-,5,13.html [Retrieved 25 November 2015]
[38] GUS, 2015. Główny Urząd Statystyczny. Departament Rolnictwa., 2015. Pogłowie bydła i owiec według stanu w czerwcu 2015 r. http://stat.gov.pl/obszary-tematyczne/rolnictwo-lesnictwo/produkcja-zwierzeca-zwierzeta-gospodarskie/poglowie-bydla-i-owiec-wedlug-stanu-w-czerwcu-2015-r-,5,14.html [Retrieved 25 November 2015]
[39] Hadjipavlou G., Matika O., Clop A., Bishop S.C., 2008. Two single nucleotide polymorphisms in the myostatin (*GDF*8) gene have significant association with muscle depth of commercial Charollais sheep. Animal Genetics 39, 346-353.
[40] Han J., Forrest R.H., Hickford J.G.H., 2013. Genetic variations in the myostatin gene (MSTN) in New Zealand sheep breeds. Molecular Biology Reports 40, 6379-6384. DOI 10.1007/s11033-013-2752-7
[41] Han J., Forrest R.H., Sedcolec J.R., Hickford J.G.H., 2015. Myostatin (MSTN) gene haplotypes and their association with growth and carcass traits in New Zealand Romney lambs. Small Ruminant Research 127(2015), 8-19.
[42] Han J., Zhou H., Forrest R.H., Sedcole J.R., Frampton C.M., Hickford J.G.H., 2010. Effect of Myostatin (*MSTN*) g+6223G>A on Production and Carcass Traits in New Zealand Romney Sheep. Asian-Aust Journal of Animal Science 23(7), 863-866.
[43] Hartl D.L., Ruvolo M., 2012. Genetics, Eighth Edition. Analysis of Genes and Genomes p, 683-687. ISBN-13: 9781449635961
[44] Haynes F.E.M., Greenwood P.L., McDonagh M.B., McMahon C.D., Nicholas G.D., Berry C.J., Oddy V.H., 2013. Lack of association between allelic status and myostatin content in lambs with the myostatin g+6723G>A allele. Journal of Animal Science 91, 78-89. doi: 10.2527/jas.2012-5482.
[45] Hickford J.G.H., Forrest R.H., Zhou H., Fang Q., Han J., Frampton C.M., Horrell A.L., 2009. Polymorphisms in the ovine myostatin gene (MSTN) and their association with growth and carcass traits in New Zealand Romney Sheep. Animal Genetics 41, 64-72. doi:10.1111/j.1365-2052.2009.01965.x.

[46] Hope M., Haynes F., Oddy H., Koohmaraie M., Al-Owaimer A., Geesink G., 2013. The effects of the myostatin g+6723G>A mutation on carcass and meat quality of lamb. Meat Science. doi: 10.1016/j.meatsci.2013.03.029.
[47] Instytut Zootechniki, 2013. Programy Ochrony Zasobów Genetycznych Zwierząt Gospodarskich. Liczebności lokalnych ras owiec objętych programem ochrony zasobów genetycznych zwierząt (lata: 2013-2011) [Online] http://www.bioroznorodnosc.izoo.krakow.pl/owce [Retrieved 25 November 2015]
[48] Jamshidi S., Karani S., Goudarzi M., 2014. Study polymorphism myostatin gene in Mehraban's sheep using PCR-RFLP method. Science International (Lahore) 26(3), 1129-1135. ISSN 1013-5316.
[49] Johnson P.L., Dodds K.G., Bain W.E., Greer G.J., McLean N.J., McLaren R.J., Galloway S.M., van Stijn T.C., McEwan J.C., 2009. Investigations into the GDF8 *g+6723G-A* polymorphism in New Zealand Texel sheep. Journal of Animal Science 87, 1856-1864.
[50] Kakavas VK., Plageras P., Vlachos T.A., Papaioannou A., Noulas V.A., 2008. PCR–SSCP: A Method for the Molecular Analysis of Genetic Diseases. Molecular Biotechnology 38, 155-163. DOI 10.1007/s12033-007-9006-7.
[51] Kambadur R., Sharma M., Smith T.P., Bass J.J., 1997. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Research 7, 910-916.
[52] Karim L., Coppieters W., Grobet L., Georges M., Valentini A., 2000. Convenient genotyping of six myostatin mutations causing double-muscling in cattle using a multiplex oligonucleotide ligation assay. Animal Genetics 31(6), 396-399.
[53] Kawęcka A., Sikora J. 2012. Owce [in] Polskie rasy zachowawcze. Atlas zwierząt gospodarskich objętych programem ochrony w Polsce. Red. Krupiński J. Instytut Zootechniki. Państwowy instytut Badawczy. Kraków 2012, ISBN 83-60127-67-0.
[54] Kemaladewi D.U., Hoogaars W.M.H., van Heiningen S.H., Terlouw S., De Gorter D.J.J., den Dunnen J.T., van Ommen G.J.B., Aartsma-Rus A., ten Dijke P., Hoen P.A.C., 2011. Dual exon skipping in myostatin and dystrophin for Duchenne muscular dystrophy for Duchenne muscular dystrophy. BMC Medical Genomics 4, 36. doi:10.1186/1755-8794-4-36.
[55] Kijas J.W., McCulloch R., Edwards J.E.H., Oddy V.H., Lee S. H. van der Werf J., 2007. Evidence for multiple alleles effecting muscling and fatness at the *Ovine* GDF8 locus. BMC Genetics 8, 80-91.
[56] Krupiński J., 2008. Ochrona zasobów genetycznych zwierząt w Polsce Wiadomości Zootechniczne, Zeszyt specjalny 1(256), 1-10.

[57] Langley B., Thomas M., Bishop A., Sharma M., Gilmour S., Kambadur R., 2002. Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. The Journal of Biological Chemistry 277(51), 49831-40.
[58] Laudowicz. A., 1997. Owce w Polsce album. Sheep breeds in Poland. Red. Laudowicz A., Zrzeszenie Związków Hodowców Owiec w Polsce.
[59] Lee S.J., 2004. Regulation of muscle mass by myostatin. Annual Review of Cell and Developmental Biology 20, 61-86.
[60] Lee S.J., Mcpherron A.C., 1999. Myostatin and the control of skeletal muscle mass: Commentary. Current Opinion in Genetics & Development 9 (5), 604-607.
[61] Lee S.J., McPherron A.C., 2001. Regulation of myostatin activity and muscle growth. Proceedings of the National Academy of Sciences 98(16), 9306-9311.
[62] Li Z., Zhao B., Kim Y.S., Hu C.Y., Yang J., 2010. Administration of a mutated myostatin propeptide to neonatal mice significantly enhances skeletal muscle growth. Molecular Reproduction and Development 77, 76-82.
[63] Lipecka C., Gruszecki T.M., 2006a. Rasy zwierząt w Polsce – owce. Owca kamieniecka. Medycyna weterynaryjna 62(9), 3.
[64] Lipecka C., Gruszecki T.M., 2006b. Rasy zwierząt w Polsce – owce. Owca Pomeranian. Medycyna weterynaryjna 62(10), 3.
[65] Lipecka C., Gruszecki T.M., 2006c. Rasy zwierząt w Polsce – owce. Merynos odmiany barwnej. Medycyna weterynaryjna 62(4), 3.
[66] Manso T., Mantecón A.R., Lavin P., Garadez F.J., Pebláez R., Ovejero F.J., 1996. Effect of level of intake during the milk-feeding period on post-weaning growth in lambs. Animal Feed Science and Technology 5, 317-325.
[67] Masri A.Y., Lambe N.R., Macfarlane J.M., Brotherstone S., Haresign W., Bünger L., 2011b. Evaluating the effects of a single copy of a mutation in the myostatin gene (c.*1232 GNA) on carcass traits in crossbred lambs. Meat Science 87, 412-418. doi:10.1016/j.meatsci.2010.11.019.
[68] Masri A.Y., Lambe N.R., Macfarlane J.M., Brotherstone S., Haresign W., Bünger L., 2011a. Evaluating the effects of the c.*1232G>A mutation and TM-QTL in Texel ×Welsh Mountain lambs using ultrasound and video image analyses. Small Ruminant Research 99, 99-109. doi:10.1016/j.smallrumres.2011.03.047.
[69] McCroskery S., Thomas M., Maxwell L., Sharma M., Kambadur R., 2003. Myostatin negatively regulates satellite cell activation and self-renewal. The Journal of Cell Biology 162(6), 1135-47.
[70] McPherron A.C., Lawler A.M. Lee S.J., 1997. Regulation of skeletal muscle mass in mice by a new TGF-p superfamily member. Nature 387, 83-90.

[71] Miar Y., Salehi A., Aleyasin A., Raoufzadeh S., 2011. Study of polymorphism in myostatin gene in Chaal, Zel and Zandi Iranian sheep Breeds. Iranian Journal of Animal Production 13(1), 33-40. (abstract) http://www.researchgate.net/publication/260797595_Study_of_polymorphism_in_myostatin_gene_in_Chaal_Zel_and_Zandi_Iranian_sheep_Breeds [Retrieved 25 November 2015]
[72] Milewski S., 2006. Walory prozdrowotne produktów owczarskich. Medycyna weterynaryjna 62(5), 516-519.
[73] Milewski S., 2014. Charakterystyka i znaczenie owiec kamienieckich w świetle wyników badań naukowych. Przegląd hodowlany 1, 17-19.
[74] MyoMAX, 2016. http://genetics.zoetis.com/australia/products/sheep/myomax.aspx [Retrieved 2 February 2016]
[75] NCBI, 2015, www.ncbi.nlm.nih.gov [Retrieved 25 November 2015]
[76] Nicholas F.W., 2010. Introduction to veterinary genetics. Blackwell Publishing p, 216-229. ISBN: 978-1-4051-6832-8.
[77] Orita M., Iwahana H., Kanazawa H., Hayashi K., Sekiya T., 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proceedings of the National Academy of Sciences USA 86(8), 2766-2770.
[78] Pakulski T., 2010. Program ochrony zasobów genetycznych oraz wykorzystania produkcyjnego i badawczego merynosa odmiany barwnej. Wiadomości Zootechniczne R. XLVIII 2-3, 30-33.
[79] Pas Te M.F.W., Everts M.E., Haagsman H.P., 2004. Role of myostatin in muscle growth. (in) Muscle Development of Livestock Animals: Physiology, Genetics, and Meat Quality. Cambridge, MA, USA, CABI Publishing p, 297-316.
[80] Piwczyński D., Mroczkowski S., 2005. Wpływ płci i typu urodzenia na masę ciała jagniąt rasy merynos polski. Prace Wydziału Nauk Przyrodniczych. Seria B. Prace Komisji Nauk Rolniczych i Biologicznych 41(55), 137-141.
[81] Rasmussen H.B., 2012. Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP) and Gel Electrophoresis - Valuable Tool for Genotyping and Genetic Fingerprinting, Gel Electrophoresis - Principles and Basics, Sameh Magdeldin (Ed.) p, 315-334. ISBN: 978-953-51-0458-2, In Tech, Available from: http://www.intechopen.com/books/gel-electrophoresis-principles-and-basics/restrictionfragment-length-polymorphism-analysis-of-pcr-amplified-fragments-pcr-rflp-and-related-te
[82] Rokicki T., 2008. Uwarunkowania rozwoju produkcji owczarskiej w Polsce. Zeszyty Naukowe SGGW - Ekonomika i Organizacja Gospodarki Żywnościowej 67, 109-116.

[83] SAS Institute Inc., 2015. SAS/STAT® 9.4 User's Guide. Cary, NC: SAS Institute Inc.
[84] Schuelke M., Wagner K.R., Stolz L.E., Hubner C., Riebel T., Komen W., Braun T., Tobin J.F., Lee S-J., 2004. Myostatin Mutation Associated with Gross Muscle Hypertrophy in a Child. The New England Journal of Medicine 350, 2682-2688.
[85] Simeonova M., Todorovb N., Nedelkovb K., Kirilov A., Harmonc D.L., 2014. Influence of live weight, sex and type of birth on growth and slaughter characteristics in early weaned. Small Ruminant Research 121(2014), 188-192.
[86] Siminska E., Bernacka H., Dymek M., 2008. Reproductive performance of Pomeranian sheep in the Baldram conservation herd. Journal of Central European Agriculture 9(1), 45-50.
[87] Sjakste T., Paramonova N., Grislis Z., Trapina I., Kairisa D., 2011. Analysis of the Single-Nucleotide Polymorphism in the 50UTR and Part of Intron I of the Sheep MSTN Gene. Dna And Cell Biology 30(7), 433-444. DOI: 10.1089/dna.2010.1153.
[88] Słomski R., Szalata M., Wielgus K., 2008. Reakcja łańcuchowa polimerazy (PCR). [in]: Analiza DNA – teoria i praktyka. Pod red. Słomski R. Wydawnictwo Uniwersytetu Przyrodniczego, Poznań p, 131-139.
[89] Sumantri C., Jakaria J., Yamin M., Nuraini H., Andreas E., 2011. Identification of myostatin gene c.960delG locus polymorphism in Indonesian local sheep by using PCR-SSCP method. Journal of the Indonesian Tropical animal Agriculture 36(3), 145-151.
[90] Szczepański W., Czarniawska-Zając S., Milewski S., 2001. Hodowla i użytkowanie owiec. Przewodnik do ćwiczeń. Wydawnictwo Uniwersytety Warmińsko-Mazurskiego. Olsztyn 2001.
[91] Szymańska A., 2009. Owce na czasie … Rolniczy Magazyn Elektroniczny 31Centralna Biblioteka Rolnicza ISSN 1734-3070.
[92] Tellam R.L., Cockett N.E., Vuocolo T., Bidwell C.A., 2012. Genes contributing to genetic variation of muscling in sheep. Frontiers in Genetics 3, 164. doi.org/10.3389/fgene.2012.00164.
[93] Trukhachev V., Belyaev V., Kvochko A., Kulichenko A., Kovalev D., Pisarenko S., Volynkina A., Selionova M., Aybazov M., Shumaenko S., Omarov A., Mamontova T., Golovanova N., Yatsyk O., Krivoruchko A., 2015. Myostatin gene (MSTN) polymorphism with a negative effect on meat productivity in Dzhalginsky Merino sheep breed. Journal of Bioscience and Biotechnology 4(2), 191-199.
[94] Uniprot, 2015. http://www.uniprot.org/uniprot/O18830 [Retrieved 5 June 2015]
[95] Wiener P., Gutierrez-Gil B., 2009. Assessment of selection mapping near the myostatin gene (GDF-8) in cattle. Animal Genetics 40, 598-608.

[96] Zhou H., Hicford J.G.H., Gong H., 2007. Polymorphism of the ovine calpastatin gene. Molecular and Cellular Probes 21, 242-244.
[97] Zhou H., Hickford J.G.H., Fang Q., 2008. Variation in the coding region of the myostatin (GDF8) gene in Steep. Molecular and Cellular Probes 22, 67-68. doi:10.1016/j.mcp.2007.08.004.

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12.TABLES AND IMAGES

Table 1 The number of sheep in Poland between 2011-2015 (GUS)

	Number of sheep in the given year (thousand individuals)				
	2011	2012	2013	2014	2015
Total Sheep population	212.7	218.5	223.1	222.8	227.7
Ewes	143.7	134.7	142.3	133.4	147.0

Table 2 The number of different local sheep breeds subjected to the Genetic Resources Conservation Programmes (2008-2013) (Instytut Zootechniki, 2013)

Sheep breed	**2008**		**2011**		**2012**		**2013**	
	Herds	**Sheep**	**Herds**	**Sheep**	**Herds**	**Sheep**	**Herds**	**Sheep**
Coloured Merino	1	90	4	218	5	260	6	344
Kamieniec	22	932	34	2.015	37	2875	42	3.388
Pomeranian	140	5.266	121	6.489	118	7.751	96	6.511

Table 3 Characteristics of performance traits of Kamieniec, Pomeranian and Coloured Polish Merino Sheep (Kawęcka and Sikora, 2012)

Trait	Breed		
	Kamieniec	**Pomeranian**	**Coloured Polish Merino**
Adult rams – body weight [kg]	90-110	80-110	80−100
Adult ewes – body weight [kg]	60-70	65-75	55−65
Fertility [%]	120	115	115
Prolificacy [%]	140	140	135
Adult rams – weight [kg]	90-110	80-110	80−100

Table 4 The number of sheep (n) in relation to sexes and type of birth throughout different sheep breeds

Factor		Number of sheep (n)			Total %
		Kamieniec	**Coloured Polish Merino**	**Pomeranian**	
Sex	Female	50	45	50	48.82
	Male	50	52	50	54.18
Total		100	97	100	100
Type of birth	Twin	61	68	20	50.18
	Singleton	39	29	80	49.83
Total		100	97	100	100

Table 5 Primer characteristics depending on performed PCR

PCR name	Primers name	Application of PCR products	Sequence 5'to 3'	Tm [ºC]*	PCR product lengths [bp]	Methodology Reference
MioClop PCR	**MioClop_F**	RFLP	TTTGGTATATTTTTACAGTAAGGAC	49.5	1.003	Clop *et al.*, 2006
	MioClop_R		TAAATAGTGTTGCACTTAAGGATTC	51.1		
Hickford PCR	**Hickford_F**	SSCP	GAAACGGTCATTACCATGC	48.9	414	Hickford *et al*., 2009
	Hickford_R		CATATTTCAGGCAACCAAATG	48.5		

* According to the primer specification provided by Genomed S.A.

Table 6 The composition of MioClop and Hickford PCR reaction mixtures (Grochowska, 2016)

Ingredient	Volume per one sample (µl)	
	MioClop PCR	**Hickford PCR**
DNA (50 ng/ µl)	2	2
H2O	13.3	14.5
Dream TaqBuffer (includes 20 mM MgCl2)	2	2
Primer F (10 pmol/ µl)	1	0.5
Primer R(10 pmol/ µl)	1	0.5
dNTP (10mM)	0.5	0.3
Dream Taq polymerase (5u/ µl)	0.2	0.2

Table 7 Conditions for MioClop and Hickford PCR reactions (Grochowska, 2016)

PCR name	PCR programme				Intended for
	Step	**Temperature [ºC]**	**Time [s]**	**Cycles number**	
MioClop	**Initial denaturation**	94°C	300	1	**RFLP**
	Denaturation	94°C	30	35	
	Annealing	59°C	30		
	Elongation	72°C	60		
	Final elongation	72°C	300	1	
Hickford	**Initial denaturation**	94°C	120	1	**SSCP**
	Denaturation	94°C	30	35	
	Annealing	56°C	30		
	Elongation	72°C	30		
	Final elongation	72°C	300	1	

Table 8 Composition of reaction mixture and conditions for enzymatic hydrolysis of MioClop PCR products (Grochowska, 2016)

Component	Volume per one sample (µl)	Temperature [ºC]	Incubation time [h]
H2O	0.3	37 ºC	4
Buffer 1 (New England BioLabs)	1.2		
***Hpy*CH4 (10U/ µl) (New England BioLabs)**	0.5		
MioClop PCR product	10		

Table 9 Frequencies [%] of myostatin genotypes in position c.*1232G>A according to sheep breed

Genotype	Breed		
	Kamieniec	**Coloured Merino**	**Pomeranian**
AA	6.00	0.00	6.00
AG	21.00	0.00	56.00
GG	73.00	100.00	38.00
P_F	< 0.0001		

P_F according to chi^2 test, that was used in order to verify whether the differences in frequencies of myostatin genotypes between breeds were significant

Table 10 Frequencies [%] of myostatin alleles in position c.*1232G>A according to sheep breeds

Allele	Kamieniec	Coloured Merino	Pomeranian
A	16.50	0.00	34.00
G	83.50	100.00	66.00

Table 11 Frequencies [%] of myostatin genotypes detected by SSCP in intron 1 according to sheep breed

Genotype	Breed		
	Kamieniec	**Coloured Merino**	**Pomeranian**
A_1B_1	51.00	1.00	8.00
B_1B_1	20.00	0.00	0.00
A_1A_1	25.00	79.40	89.00
A_1C_1	1.00	4.10	1.00
B_1C_1	2.00	0.00	0.00
A_1D_1	1.00	0.00	1.00
A_1E_1	0.00	11.30	1.00
A_1F_1	0.00	4.10	0.00
P_F	< 0.0001		

P_F according to chi^2 test, that was used in order to verify whether the differences in frequencies of myostatin genotypes between breeds were significant

Table 12 Frequencies [%] of myostatin alleles detected by SSCP in intron 1 according to sheep breeds

Allele	Kamieniec	Coloured Merino	Pomeranian
A_1	51.5	89.69	94.5
B_1	46.5	0.52	4
C_1	1.5	2.06	0.5
D_1	0.5	0.00	0.5
E_1	0	5.67	0.5
F_1	0	2.06	0

Table 13 Comparison of the nucleotide sequences in each of the polymorphic sites in intron 1 of myostatin gene between the alleles obtained in the present studies and those obtained by Hickford *et al*. (2009)

Nucleotide position (from the beginning of the gene)	**Alleles detected in this study**						**Allele detected by Hickford *et al.*, 2009**				
	A_1	**B_1**	**C_1**	**D_1**	**E_1**	**F_1**	**A**	**B**	**C**	**D**	**E**
391	G	T	T	T	T	T	G	T	T	T	T
474	C	C	C	C	C	T	T	T	T	T	T
614	T	C	T	T	T	T	T	C	T	T	T
616	G	A	A	A	G	G	G	A	A	A	G
619	T	T	T	C	T	T	T	T	T	C	T
622	T	T	C	T	T	T	T	T	C	T	T
632	G	T	T	T	T	T	G	T	T	T	T
696	C	C	T	C	C	C	C	C	T	C	C

A – adenine, T – thymine, C- cytosine, G –guanine,

Table 14 Nucleotide distribution in polymorphic sites in intron 1 in relation to genotypes detected by SSCP

Genotype	**Position in GDF8 gene sequence (nt) starting from the beginning of the gene**							
	391	**474**	**614**	**616**	**619**	**622**	**632**	**696**
Reference	GG	CC	TT	GG	TT	TT	GG	CC
A_1B_1	GT	CC	TC	AG	TT	TT	GT	CC
B_1B_1	TT	CC	CC	AA	TT	TT	TT	CC
A_1A_1	GG	CC	TT	GG	TT	TT	GG	CC
A_1C_1	GT	CC	TT	AG	TT	CT	GT	CT
B_1C_1	TT	CC	CT	AA	TT	CT	TT	CT
A_1D_1	GT	CC	TT	AG	CT	TT	GT	CC
A_1E_1	GT	CC	TT	GG	TT	TT	GT	CC
A_1F_1	GT	CT	TT	GG	TT	TT	GT	CC

A – adenine, T – thymine, C- cytosine, G -guanine

Table 15 Lamb's statistical characteristics in terms of different body weights depending on myostatin genotypes detected by RFLP

Trait	Measures	Genotype		
		AA	**AG**	**GG**
Kamieniec				
	N	**6**	**21**	**73**
Body weight at 2. day	Mean [kg]	4.93	4.92	4.91
	Std	0.75	0.48	0.61
	CV	15.10	9.78	12.41
Body weight at 56 days	Mean [kg]	17.29	18.60	16.46
	Std	3.71	3.89	3.71
	CV	21.45	20.90	22.55
Daily gains between 2. and 56. day	Mean [g]	228.80	253.20	213.80
	Std	57.13	66.02	62.74
	CV	24.97	26.07	29.35
Pomeranian				
	N	**6**	**56**	**38**
Body weight at 2. day	Mean	4.07	4.19	4.16
	Std	0.23	0.49	0.60
	CV	5.75	11.63	14.38
Body weight at 56 days	Mean	20.27	21.44	21.54
	Std	3.95	3.72	3.30
	CV	19.48	17.33	15.30
Daily gains between 2. and 56. day	Mean	300.30	319.50	322.00
	Std	72.16	68.99	56.92
	CV	24.03	21.59	17.68
Coloured Merino				
	N	**0**	**0**	**97**
Body weight at 2. day	Mean	.	.	4.86
	Std	.	.	0.87
	CV	.	.	17.96
Body weight at 56 days	Mean	.	.	19.48
	Std	.	.	3.06
	CV	.	.	15.72
Daily gains between 2. and 56. day	Mean	.	.	270.80
	Std	.	.	50.12
	CV	.	.	18.51
Body weight at 30 days	Mean	.	.	12.31
	Std	.	.	2.28
	CV	.	.	18.55
Body weight at 70 days	Mean	.	.	23.66
	Std	.	.	3.42
	CV	.	.	14.45

Table 16 Statistical characteristics of Kamieniec lambs in terms of different body weights depending on myostatin genotypes detected by SSCP

Trait	Measures	Genotype		
		A_1B_1 N=51	**B_1B_1 N=20**	**A_1A_1 N=25**
Body weight at 2. day	Mean	4.95	4.87	4.82
	Std	0.53	0.66	0.60
	CV	10.76	13.46	12.38
Body weight at 56 days	Mean	17.11	16.95	16.40
	Std	3.52	3.68	4.25
	CV	20.54	21.74	25.91
Daily gains between 2. and 56. day	Mean	225.30	223.70	214.40
	Std	59.30	63.59	71.66
	CV	26.32	28.43	33.42

Table 17 Statistical characteristics of Pomeranian lambs in terms of different body weights depending on myostatin genotypes detected by SSCP

Trait	Measures	Genotype	
		A_1B_1 N=8	**A_1A_1 N=89**
Body weight at 2. day	Mean	4.06	4.18
	Std	0.29	0.54
	CV	7.08	12.84
Body weight at 56 days	Mean	19.9	21.61
	Std	2.15	3.65
	CV	10.79	16.89
Daily gains between 2. and 56. day	Mean	293.20	322.90
	Std	38.91	66.16
	CV	13.27	20.49

Table 18 Characteristics of Coloured Merino lambs in terms of different body weights depending on myostatin genotypes detected by SSCP

Trait	Measures	Genotype	
		A_1A_1 N=77	**A_1E_1 N=11**
Body weight at 2. day	Mean	4.76	5.13
	Std	0.85	0.94
	CV	17.86	18.34
Body weight at 56 days	Mean	19.46	19.56
	Std	2.92	4.62
	CV	15.03	23.61
Daily gains between 2. and 56. day	Mean	272.20	267.20
	Std	47.24	78.92
	CV	17.35	29.54
Body weight at 30 days	Mean	12.28	12.26
	Std	2.19	3.27
	CV	17.84	26.69
Body weight at 70 days	Mean	23.63	23.41
	Std	3.30	4.66
	CV	13.98	19.89

Table 19a The influence of sex and type of birth on lamb body weights in Kamieniec and Pomeranian sheep breeds

Trait	Measures	Sex		Type of birth	
		Female	Male	Singleton	Twin
Kamieniec					
	N	**50**	**50**	**39**	**61**
Body weight at 2. day	Mean[kg]	4.72B	5.11A	5.28A	4.68B
	Std	0.58	0.54	0.54	0.50
	CV	12.18	10.49	10.18	10.61
Body weight at 56 days	Mean[kg]	15.66B	18.25A	19.82A	15.12B
	Std	3.07	4.06	3.68	2.58
	CV	19.60	22.26	18.55	17.09
Daily gains between 2. and 56. day	Mean [g]	202.60B	243.30A	269.30A	193.30B
	Std	51.63	70.06	63.77	44.86
	CV	25.48	28.80	23.68	23.20
Pomeranian					
	N	**50**	**50**	**80**	**20**
Body weight at 2. day	Mean[kg]	3.96B	4.39A	4.27A	3.79B
	Std	0.38	0.55	0.50	0.40
	CV	9.62	12.57	11.74	10.68
Body weight at 56 days	Mean[kg]	21.29	21.53	22.26A	18.02B
	Std	3.69	3.44	3.22	2.74
	CV	17.32	15.98	14.46	15.22
Daily gains between 2. and 56. day	Mean [g]	321.10	317.40	333.20A	263.60B
	Std	68.43	60.66	59.53	52.66
	CV	21.31	19.11	17.87	19.98

AB – means marked with the same capital letters are not statistically different (P=<0.01)

ab - means marked with the identical lower case are not statistically different (P=<0.05)

Table 19b The influence of sex and type of birth on lamb body weights in Coloured Polish Merino sheep breed

Trait	Measures	Sex		Type of birth	
		Female	**Male**	**Singleton**	**Twin**
Coloured Merino					
	N	**45**	**52**	**29**	**68**
Body weight at 2. day	Mean[kg]	4.63b	5.06a	5.44A	4.61B
	Std	0.70	0.96	0.90	0.74
	CV	15.14	18.97	16.47	16.05
Body weight at 56 days	Mean[kg]	18.70b	20.16a	21.64A	18.56B
	Std	2.77	3.17	3.36	2.42
	CV	14.82	15.70	15.50	13.03
Daily gains between 2. and 56. day	Mean [g]	260.50b	271.40a	297.70A	252.90B
	Std	48.69	64.68	59.96	51.70
	CV	18.69	23.83	20.14	20.44
Body weight at 30 days	Mean[kg]	11.79b	12.76a	14.2A	11.5B
	Std	2.10	2.36	2.44	1.66
	CV	17.78	18.51	17.21	14.45
Body weight at 70days	Mean[kg]	22.65b	24.53a	25.2A	23B
	Std	2.967	3.57	3.98	2.941
	CV	13.10	14.55	15.81	12.79

AB – means marked with the same capital letters are not statistically different (P=<0.01)

ab - means marked with the identical lower case are not statistically different (P=<0.05)

Table 20a The influence of the genotype detected by SSCP and type of birth on different carcass cuts of Coloured Polish Merino rams

Trait	Measures	Genotype detected by SSCP		Type of birth	
		A_1A_1	A_1E_1	Singleton	Twin
		N=22	N=6	N=8	N=20
Front part	Mean [g]	2738.00	2722.00	2961.00	2644.00
	Std	428.40	657.40	641.40	367.30
	CV	15.65	24.15	21.66	13.89
Middle part	Mean [g]	1807.00	1786.00	1981.00	1731.00
	Std	282.90	534.80	409.20	288.60
	CV	15.66	29.94	20.65	16.67
Hind part	Mean [g]	2281.00	2338.00	2509.00	2208.00
	Std	309.40	629.30	539.70	276.10
	CV	13.56	26.91	21.51	12.51
Scrag	Mean [g]	364.1	374.20	395.60	354.50
	Std	74.04	69.38	95.07	59.27
	CV	20.34	18.54	24.03	16.72
Foreshank	Mean [g]	313.60	327.50	334.40	309.50
	Std	41.21	70.48	71.98	33.71
	CV	13.14	21.52	21.53	10.89
Neck	Mean [g]	568.20	559.20	618.80	545.30
	Std	97.73	121.50	106.90	92.88
	CV	17.20	21.72	17.27	17.04
Shoulder	Mean [g]	1096.00	1090.00	1201.00	1053.00
	Std	166.50	313.90	274.00	149.50
	CV	15.19	28.80	22.82	14.20
Breast	Mean [g]	1046.00	1058.00	1133.00	1015.00
	Std	174.90	317.90	285.80	161.60
	CV	16.72	30.06	25.22	15.93
Entrecote	Mean [g]	525.50	507.50	582.50a	497.30b
	Std	85.56	143.10	116.10	80.04
	CV	16.28	28.19	19.93	16.10
Loin	Mean [g]	568.40	535.00	611.30	541.30
	Std	98.71	163.60	114.60	108.50
	CV	17.37	30.57	18.74	20.05
Sirloin	Mean [g]	61.59	64.17	67.50	60.00
	Std	12.19	17.72	15.81	11.81
	CV	19.79	27.62	23.42	19.68

AB – means marked with the same capital letters are not statistically different (P=<0.01)

ab - means marked with the identical lower case are not statistically different (P=<0.05)

Table 20b The influence of the genotype detected by SSCP and type of birth on different carcass cuts of Coloured Polish Merino rams

Trait	Measures	Genotype detected by SSCP		Type of birth	
		A_1A_1	A_1E_1	Singleton	Twin
		N=22	N=6	N=8	N=20
Hindshank	Mean [g]	414.10	432.50	443.80	407.80
	Std	48.44	113.20	96.24	47.39
	CV	11.70	26.17	21.69	11.62
Leg	Mean [g]	1869.00	1909.00	2067.00	1802.00
	Std	263.10	519.40	444.80	232.00
	CV	14.08	27.20	21.52	12.88
Leg prior to dissection	Mean [g]	1856.00	1894.00	2053.00	1789.00
	Std	261.90	516.70	441.70	231.40
	CV	14.11	27.28	21.51	12.93
Muscle tissue in leg	Mean [g]	1348.00	1384.00	1471.00	1310.00
	Std	180.40	366.10	315.00	165.10
	CV	13.38	26.45	21.42	12.60
Fat tissue in leg	Mean [g]	232.70	220.00	284.40A	208.30B
	Std	68.85	84.62	80.38	55.14
	CV	29.58	38.46	28.26	26.48
Bone tissue in leg	Mean [g]	275.00	290.00	298.10	270.30
	Std	37.13	73.35	68.50	32.14
	CV	13.50	25.29	22.98	11.89
Muscle tissue in leg [%]	Mean	72.76	73.20	71.71	73.31
	Std	2.30	1.42	2.43	1.86
	CV	3.16	1.94	3.39	2.54
Fat tissue in leg [%]	Mean	12.37	11.41	13.77a	11.53b
	Std	2.48	1.54	2.02	2.16
	CV	20.05	13.50	14.68	18.70
Bone tissue in leg [%]	Mean	14.86	15.39	14.53	15.16
	Std	1.14	0.90	1.33	0.90
	CV	7.69	5.83	9.18	6.44

AB – means marked with the same capital letters are not statistically different (P=<0.01)

ab - means marked with the identical lower case are not statistically different (P=<0.05)

Table 21 The influence of myostatin genotypes on different body weights (Model 2)

Trait	Measures	Genotype detected by RFLP			Genotype detected by SSCP			
		AA	**AG**	**GG**	**A_1B_1**	**B_1B_1**	**A_1A_1**	**A_1E_1**
		N=12	**N=77**	**N=208**	**N=59**	**N=20**	**N=191**	**N=11**
Body weight at 2. day	Mean [kg]	4.50ab	4.39b	4.75a	4.83AB	4.87AB	4.50B	5.13A
	Std	0.69	0.58	0.79	0.59	0.66	0.75	0.94
	CV	15.43	13.30	16.65	12.21	13.46	16.62	18.34
Body weight at 56 days	Mean [kg]	18.78b	20.66a	18.80b	17.49BC	16.95C	20.06A	19.56AB
	Std	3.97	3.95	3.82	3.48	3.68	3.86	4.62
	CV	21.14	19.11	20.34	19.92	21.74	19.24	23.61
Daily gains between 2. and 56. day	Mean [g]	264.60B	301.40A	258.10B	234.50BC	223.70C	287.60A	255.20AB
	Std	72.43	73.98	70.50	61.35	63.59	73.13	77.76
	CV	27.38	24.55	27.32	26.16	28.43	25.43	30.47

AB – means marked with the same capital letters are not statistically different (P=<0,01)

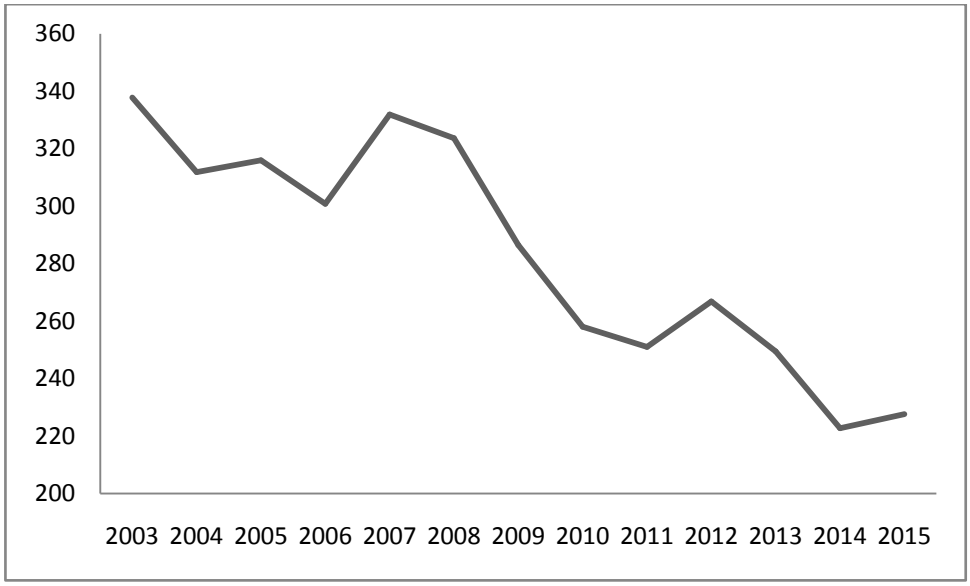
ab - means marked with the identical lower case are not statistically different (P=<0,05)

Table 22 The influence of sex, type of birth and breed on different body weights (Model 2)

Trait	Measures	Sex		Type of birth		Breed		
		Female N=145	**Male N=152**	**Singleton N=148**	**Twin N=149**	**Kamieniec N=100**	**Coloured Merino N=97**	**Pomeranian N=100**
Body weight at 2. day	Mean [kg]	4.43A	4.86B	4.76B	4.53A	4.92A	4.86A	4.17B
	Std	0.66	0.78	0.81	0.68	0.59	0.87	0.52
	CV	14.83	16.11	17.00	14.92	11.95	17.96	12.44
Body weight at 56 days	Mean [kg]	18.55A	19.98B	21.49B	17.08A	16.96C	19.48B	21.41A
	Std	3.96	3.79	3.50	3.00	3.81	3.06	3.55
	CV	21.36	18.97	16.29	17.59	22.48	15.72	16.58
Daily gains between 2. and 56. day	Mean [g]	261.40A	277.30B	309.40B	230.00A	223.00C	266.30B	319.30A
	Std	75.25	71.60	66.35	57.71	64.55	57.78	64.36
	CV	28.78	25.82	21.45	25.09	28.95	21.70	20.16

AB – means marked with the same capital letters are not statistically different (P=<0.01)

ab - means marked with the identical lower case are not statistically different (P=<0.05)



Graph 1 The change in the total number of sheep in Poland according to the year (in thousands) (GUS, 2015)

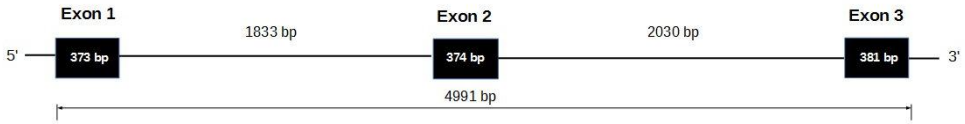


Fig. 1 Myostatin gene structure (own elaboration)

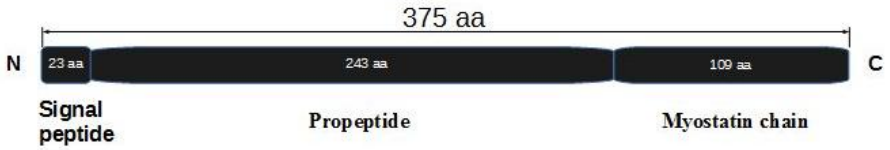


Fig. 2 The structure of myostatin protein (own elaboration)

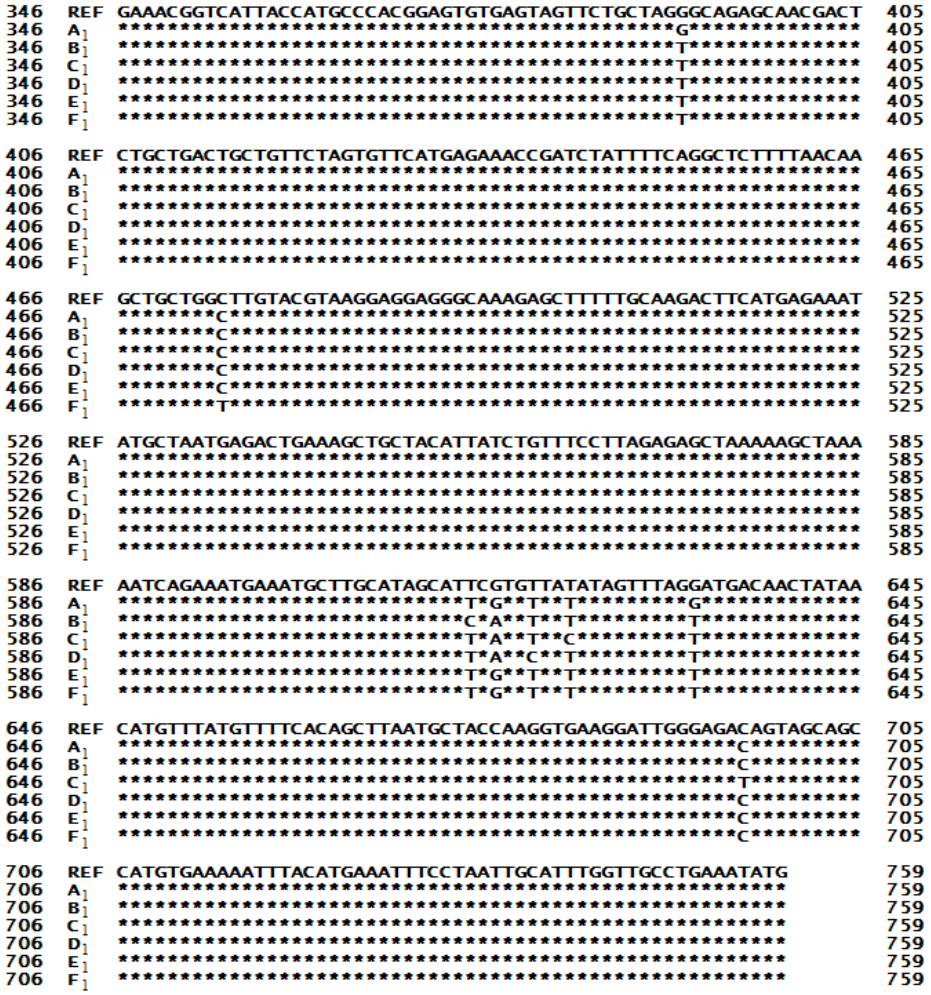


Fig. 3 Nucleotide sequences of the ovine *GDF*8 alleles detected by SSCP in intron 1. The sequences start at the 346th nucleotide of myostatin gene. Reference sequence (REF) is sourced from the Oar_v3.1 reference genome (NCBI Reference Sequence: NC_019459.1). Sequences were aligned using MUSCLE 3.8 engine (Edgar, 2014a,b). Nucleotide identical to REF are presented as asterisks (*), A – adenine, T – thymine, C- cytosine, G –guanine.

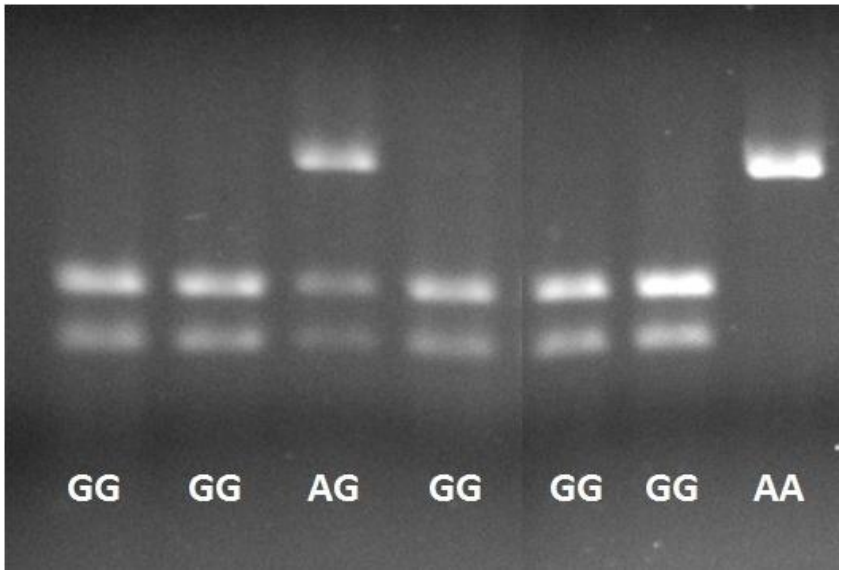


Image 1 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) band patterns obtained by digesting 1.003 bp DNA fragment of ovine *GDF*8 gene with the use of *Hpy*CH4IV restriction enzyme. Electrophoresis was carried out on 2% agarose gel with the additive of Midori Green DNA Stain for 90 min at constant 120V. The GG genotype presents two fragments (270 and 733 bp), the heterozygote (AG) three fragments (270, 733 and 1.003 bp). while homozygote for allele A presents only one DNA fragment (1.003 bp).

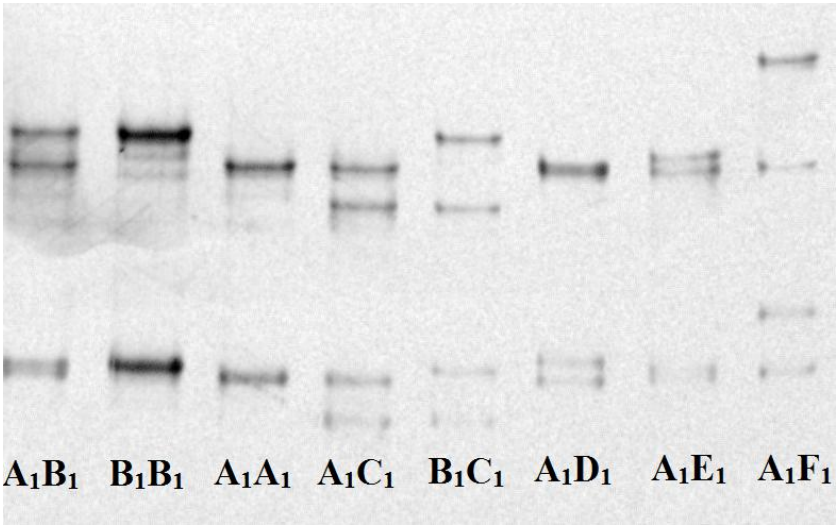


Image 2 Single-strand conformational polymorphism of PCR fragments spanning 414-bp fragment of intron 1 of ovine *GDF*8 gene. Separation was run in 8.5% polyacrylamide gel, for 21.5 hours, at 14ºC. 380V (Grochowska, 2016). Eight different genotypes and six alleles were detected.