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Meat quality – Genetic background and methods of its analysis

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Abstract: Growing consumer awareness is forcing food producers to supply raw material and products of increasingly high quality and health-promoting properties. Knowledge of the genetic background of quality characteristics is taking on great importance, enabling selection based on molecular markers. The increasing throughput of molecular techniques, in combination with an expanding bioinformatics infrastructure, is leading to continual improvement in understanding of the molecular mechanisms influencing meat quality. This has resulted in the identification of polymorphic nucleotides [single nucleotide polymorphisms (SNPs)] showing a relationship with meat characteristics such as tenderness [polymorphism in the calpain (*CAPN*) and calpastatin (*CAST*) genes], marbling [diacylglycerol o-acyltransferase 1 (*DGAT1*)], colour, pH and water-holding capacity (WHC) [*CAST*, stearoyl-CoA desaturase (*SCD*) and others], and fatty acid profile (*SCD1*). An increasingly wide range of methods is used for analysis, from techniques based on amplification of nucleic acids [polymerase chain reaction-restriction fragment length polymorphism (PCR--RFLP) and amplification refractory mutation system-PCR (ARMS-PCR)] through Sanger sequencing to high-throughput next-generation sequencing (NGS) techniques. This paper is a review of the literature on polymorphism of genes determining the quality characteristics of meat and molecular methods used to detect them.

Keywords: meat tenderness; fatty acid profile; molecular markers; SNP; molecular techniques

The meat industry is an important branch of the agri-food sector. The high competitiveness of today's market and growing consumer demands are forcing producers to provide products of the highest possible quality. Meat can be used directly as a food product or can be a raw material for further processing. Whatever its ultimate purpose, the first link in the chain is the livestock breeder, whose task is to obtain animals for slaughter that will provide raw meat with the appropriate parameters. This goal can be met by ensuring optimal farming conditions, but also through appropriate selection and mating of animals.

Meat quality is an important aspect of contemporary food technology and animal husbandry. The growing interest in this subject is confirmed by the increase in the number of articles with the phrase 'meat quality' in the PubMed database: 401 articles containing this phrase were found in 2010, and 1 486 in 2019.

Selection assisted by molecular markers is an increasingly widely raised issue. The possibilities offered by molecular biology techniques can be used to analyse entire genomes in search of polymorphisms that influence production traits, and thus the economics of livestock farming. There are reports of a relationship between single nucleotide polymorphisms (SNPs) and meat quality characteristics such as juiciness (Corva et al. 2007; Gill et al. 2009), intramuscular fat content (Bonilla et al. 2010), changes in colour during storage (Li et al. 2013), and fatty acid profile (Taniguchi et al. 2004; Sevane et al. 2014). A better understanding of the

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genetic determinants of traits of importance for livestock farmers, processors, and consumers is a key to optimisation of breeding processes and supply of products with appropriate sensory and health-promoting properties to the market (Picard et al. 2015).

GENETIC BASIS OF MEAT QUALITY CHARACTERISTICS

Traits determining the attractiveness of meat for consumers and processors can be considered at various levels, from the chemical composition and biochemical parameters of raw meat to sensory properties resulting from a combination of multiple factors, both genetic and environmental ones. The genetic component is significant because it contains information about the animal's predispositions to develop specific features which the breeder can influence through appropriate breeding work [Table S1; for Table S1 see electronic supplementary material (ESM)].

Meat colour. One of the characteristics of interest to the raw meat consumer is its colour. Li et al. (2013) found that SNP located at position 528 of the leptin gene (*LEP*) promoter region was statistically significantly associated with colour saturation and deoxymyoglobin (DeoxyMb) content in meat after six days of exposure to air. In the following text, letters in italics refer to the nucleotide sequence, letters written in regular text refer to the amino acid sequence. The authors showed that colour saturation in homozygous thymine-thymine (*TT*) individuals is lower than in the case of heterozygous cytosine-thymine (*CT*) and homozygous *CC* genotypes (by over 4.7% and over 6.2%, respectively). The inverse relationship was observed for DeoxyMb content, as *TT* homozygotes had more than 1.5% higher content of DeoxyMb than heterozygous *CT* and homozygous *CC* individuals. The same authors also reported differences in the a^* and b^* colour components and in the content of oxymyoglobin (OxyMb) on the sixth day between alleles of the stearoyl-CoA desaturase-1 (*SCD1*) gene. In individuals with the guanine-adenine (*GA*) and *AA* genotypes, the values for a^*, b^*, saturation and OxyMb content were higher than in the case of the homozygous *GG* genotype. Genes influencing meat colour include those coding for calpain (*CAPN1*) and calpastatin (*CAST*) and the myoglobin gene (*MB*). Polymorphisms in the *MB* gene show a particularly strong link to colour. Castro et al. (2016) identified two SNPs that were statistically significantly associated with colour parameters; the meat of 5870*TT* (presence of thymines in intron 1 at position 5 870) and MB11732*CC* [presence of cytosines in 3'untranslated regions (3'UTR) region at position 11 732] homozygous individuals had a more distinct and saturated red colour with a lower hue angle (Castro et al. 2016).

Muscle mass and meat tenderness. It seems important for breeders to acquire animals with the highest possible lean meat content. Research indicates a significant relationship between muscle mass and the sequence of the myostatin gene (*MSTN*, also known as *GDF-8*), which is involved in the development of muscle tissue. Changes in the gene sequence resulting in non-functional products of expression lead to excessive muscle tissue growth (Wiener et al. 2009). Mutations in the *GDF-8* gene may take the form of indel polymorphisms, involving the insertion of an additional nucleotide or the deletion of one that naturally appears in the sequence. In the Belgian Blue breed, 11 nucleotides are deleted in exon 3, which leads to changes in the reading frame, and thus to the formation of a phenotype with the double muscling characteristic of this breed. Mutations causing double muscling can also be substitutions (replacement of the nucleotide occurring in the wild genotype with a different one, leading to a mutated allele). In Piedmontese cattle, a missense mutation leads to the replacement of the amino acid cysteine with tyrosine, which results in a phenotype characterised by muscle tissue hypertrophy and double muscling (McPherron and Lee 1997). Research results (Ngapo et al. 2002) indicate that the meat of Belgian Blue cattle with double muscling had about 35% lower collagen content than that of animals with a normal phenotype. The relationship between the genotype and collagen content was statistically significant (at $P < 0.01$ for collagen expressed as mg g^{-1} wet weight and $P < 0.001$ for collagen expressed as a percentage of fat-free dry matter).

The same researchers showed differences in the content of intramuscular fat (0.7% in the homozygote with double muscling *vs.* 1.5% in the homozygote with normal muscling, statistically significant difference at $P < 0.001$), as well as in meat tenderness, expressed as the Warner-Bratzler shear force (WBSF). In the case of raw meat, individuals with double muscling had over 50% lower WBSF than homozygotes with normal muscling (statistically significant difference at $P < 0.001$). For grilled and boiled meat, the differences in WBSF between genotypes were about 20% (Ngapo et al. 2002). Problems with parturition are an unquestionable disadvantage of cattle breeds with double muscling. The animals' greater birth weight increases the risk of complications during calving (Bellinge et al. 2005).

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Polymorphism in the *GDF-8*, with a lower phenotypic effect than in individuals with double muscling, was detected in the Limousin breed, in which replacement of cytosine with adenine at nucleotide position 282 in exon 3 led to the replacement of the amino acid leucine with phenylalanine (F94L). Animals with this substitution had better muscling, intermediate between the normal phenotype and double muscling, as well as more favourable tenderness. Lines et al. (2009) confirmed that meat from homozygous individuals with genotype *AA* (two adenines encoding leucine at both loci) contained over 14% less collagen than the meat of individuals with the wild (*CC*) genotype.

Measurement of meat tenderness by the Warner-Bratzler method with some modification showed that meat from individuals with the *AA* genotype (two adenines) was over 15% more tender on the first day after slaughter and over 12% more tender after 26 days of ageing (Lines et al. 2009). Importantly, individuals with the F94L polymorphism had a similar birth weight to those with normal muscling, which reduces the risk of problems during calving (Esmailizadeh et al. 2008).

Biochemical transformations, taking place both shortly before slaughter and during meat ageing, play an important role in determining the quality parameters of meat. The animal's genotype affects the post-mortem changes taking place in the muscles, which influence its tenderness, among other traits. The enzyme system formed by the proteolytic enzymes CAPN and CAST plays an important role in these processes (Lonergan et al. 2010). CAPNs are involved in breaking down the proteins that make up myofibrils, while CASTs function in the proteolytic system as inhibitors of CAPN activity (Calvo et al. 2014). CAPNs are enzymes whose activity is dependent on the presence of calcium ions. Of the greatest importance in determining the tenderness of meat are CAPN1 (μCAPN), which requires calcium ions in micromolar concentrations to function properly, CAPN2 (mCAPN), for which Ca^{2+} should be at the millimolar level, and CAPN3, whose activity requires nanomolar concentrations of calcium ions (Diaz et al. 2006; Lana and Zolla 2016). Meat texture is strongly linked to μCAPN, encoded by the *CAPN1* gene, and to CAST, encoded by the *CAST* gene on the seventh chromosome.

Given the biochemical role of the CAPN/CAST enzyme system in meat ageing, interest among researchers in the sequences of the genes coding for these proteins is understandable. Genetic differences in the sequences of the *CAST*, *CAPN3* and μ*CAPN* genes are usually associated with the occurrence of genotypes resulting in a phenotypic effect with better tenderness parameters, without an accompanying decrease in other important raw meat characteristics (Cafe et al. 2010a, b). Polymorphisms in the genes coding for CAPN and CAST are detected in both coding elements (exons) and non-coding elements (introns) (Allais et al. 2011). Changes in coding fragments may be synonymous, causing no changes in the amino acid sequence in the protein, or nonsynonymous, causing changes in the amino acid sequence and potentially in the protein structure and properties of the raw meat as well. Page et al. (2002, 2004) showed that polymorphisms in exons 9 and 14 of the *CAPN1* gene leading to changes in the amino acid sequence are associated with a decrease in instrumentally measured meat tenderness (Page et al. 2004). Polymorphisms at positions 316 and 530 of the amino acid sequence are distinguished as particularly important. Results reported by Page et al. (2002) on the tenderness of meat 14 days after slaughter indicate that the WBSF was on average 0.26 kg lower in the individuals with valine at position 530 and glycine at position 316 than in the individuals with isoleucine and alanine at the respective positions.

The effect of the Val530Ile polymorphism in exon 14 on meat tenderness has also been confirmed in the Korean cattle breed Hanwoo (Lee et al. 2014). Molecular modelling by the same researchers taking into account the interactions between proteins showed that the Val530Ile variant has a lower capacity to bind CAST than the wild variant. In this way the effect of CAPN is inhibited much more slowly, resulting in meat that is more tender.

Intensive research is also conducted on the second element of the enzyme system, CAST. In the sequence of the gene encoding this protein, polymorphic nucleotides showing an association with meat tenderness and juiciness have been detected in both exons and introns. Calvo et al. (2014) detected an *A* > *G* (substitution of adenine for guanine) polymorphism in exon 7 of the *CAST* gene, leading to the replacement of threonine with alanine at position 182 of the amino acid sequence. Meat from *GG* homozygotes was less tender (on average by 1.79 kg cm^{-2}) than that of heterozygous *AG* and homozygous *AA* individuals, which the authors explained as the effect of the polymorphism on the functionality of the protein and the greater stability of its binding to CAPN. Lee et al. (2014) found polymorphisms in the *CAST* gene that had both positive (*CAST*: c. 182*A* > *G*, where: notation c. – addresses to the SNPs location in cDNA sequence) and negative

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(*CAST*: c. 1985*G* > *C*) effects on the meat juiciness as assessed by a sensory panel.

Polymorphisms in the nucleotide sequence can also occur in gene regions that are not directly involved in the coding of the amino acid sequence. Polymorphisms in non-coding elements (introns) (Juszczuk-Kubiak et al. 2008) or UTR (Cheong et al. 2008) can lead to differences in regulatory element binding, and consequently to differences in messenger ribonucleic acid (mRNA) stability and expression between alleles (Lindholm-Perry et al. 2009; Juszczuk-Kubiak et al. 2010). A study on pig breeds raised in Poland, including Pulawska, Polish Landrace and Polish Large White, confirmed a relationship between polymorphisms in intron 6 of the CAST gene and pork traits such as water-holding capacity (WHC), firmness, and toughness (Ropka-Molik et al. 2014). Using the enzyme RsaI to digest the sequence of intron 6 in the CAST gene, the researchers confirmed the presence of the EE genotype in all breeds, which showed a statistically significantly lower WHC (32%) in the *longissimus dorsi* muscle than the EF heterozygote (WHC = 34.6%) and FF homozygote (WHC = 36.12%).

Polymorphisms in UTR can also be of importance for the phenotypic effect. A study by Cheong et al. (2008) in the Hanwoo breed indicates an association between polymorphism in the sequence of the 3'UTR region of the CAPN gene (*CAPN1*) and beef marbling. The c. 2 151 * 479*C* > *T* substitution showed a statistically significantly higher marbling score for the meat of *CC* homozygotes than that of *CT* heterozygotes and *TT* homozygotes. Another study, conducted on pigs of the Landrace, Large White and Pulawska breeds, showed an association between polymorphism in the 3'UTR region of the perilipin-2 gene (*PLIN2*) and the quality characteristics of meat (Polasik et al. 2019). In all breeds, the researchers confirmed the presence of the *GG* genotype, which was distinguished by a statistically significantly higher content of intramuscular fat (from 0.054% in White Large to 0.123% in Pulawska). Significant differences were also observed for WHC, as the *GG* genotype was associated with a higher (on average by 4.73%) WHC.

Lipid profile. Apart from the sensory attributes of meat, consumers are increasingly interested in its nutritional value and effect on the human body, which is largely determined by its biochemical profile. This includes individual amino acids, lipids, vitamins, and other compounds playing a role in cellular metabolism.

Particular attention is paid to fat content and fatty acid profile. The content of fat and its qualitative composition not only affect the sensory attributes of meat (lipids contain dissolved compounds responsible for flavour and aroma) and its suitability for processing, but also its health benefits. Numerous studies indicate the health-promoting effects of unsaturated fatty acids and their importance in the prevention of cardiovascular and neurodegenerative diseases (Yum et al. 2016; Fuke and Nornberg 2017; Zarate et al. 2017; Sun et al. 2018).

The results of a study by Dunner et al. (2013) provide an insight into the genetic basis of the lipid profile of meat. The authors investigated SNPs in over 200 genes involved in lipid metabolism and identified 16 SNPs that had a significant effect on the lipid composition of muscle tissue. Polymorphisms of particular importance for the lipid profile included the Ile47Thr polymorphism in the cofilin 1 (*CFL1*) gene and a polymorphism in intron 2 of the myozenin 1 (*MYOZ1*) gene, which were associated with an 8% reduction in the ratio of 18 : 2 to 18 : 3 acids. Also of importance from the consumer's point of view were differences in the nucleotide sequence of the phospholipid transfer protein (*PLTP*) gene, as the SNP in the 3'UTR region reduced the ratio of *n*-6 to *n*-3 fatty acids by 8%, and differences in the nucleotide sequence of the matrix metallopeptidase 1 (*MMP1*) gene, as polymorphism in the 3'UTR region was associated with a 14% increase in the content of docosahexaenoic acid (DHA) in the muscles.

Genes playing a key role in lipid metabolism are significantly linked to the lipid profile. One such gene is diacylglycerol O-acyltransferase 1 (*DGAT1*), which catalyses the final step of triglyceride synthesis. Polymorphisms in this gene are studied with respect to the quality characteristics of both meat (Yuan et al. 2013) and milk (Tabaran et al. 2015; Bovenhuis et al. 2016). A nonsynonymous substitution whereby alanine is replaced with lysine at position 232 is of particular importance. In the *semitendinosus* muscle of homozygous German Holstein cattle with two lysine alleles at the locus of the *DGAT1* gene, higher content of intramuscular fat was observed than in heterozygous individuals (by more than 2%) and in homozygotes containing alanine (by over 1%; $P < 0.01$) (Thaller et al. 2003). The same research team also reported that polymorphism in the *TG* gene (*C/T* polymorphism at position 1 696) coding for thyroglobulin affects the content of intramuscular fat. *TT* homozygotes of the German Holstein breed had over 2.6% more intramuscular fat in the *longissimus dorsi* muscle than *CT* heterozygotes and *CC* homozygotes (statistically significant difference at $P < 0.05$).

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A similar tendency for the effect of the *DGAT1* gene was observed in a study on a larger number of Angus animals, in which the meat of homozygous individuals with a genotype determining the occurrence of lysine contained significantly more intramuscular fat in both the *longissimus dorsi* muscle (*AA* genotype encoding lysine: 18.08%, *GC* genotype encoding alanine: 12.87%) and the *semitendinosus* muscle (*AA* genotype encoding lysine: 12.06%, *GC* genotype encoding alanine: 9.04%) (Anton et al. 2011). Statistically significant differences between the content of intramuscular fat and genotype were also noted for the leptin gene (*CC* genotype: 8.88%, *TT*: 12.52% in the *semitendinosus* muscle) and the *TG* gene (statistically significant differences in both the *longissimus dorsi* muscle and the *semitendinosus* muscle).

Another gene that plays an important role in lipid metabolism is *SCD1*, which codes for an enzyme responsible for converting saturated fatty acids (SFA) to monounsaturated fatty acids (MUFA) in the fat cells of mammals. Significantly higher MUFA content was shown in steers of the Japanese Black breed with a nonsynonymous substitution leading to the replacement of valine with alanine. The meat of homozygous AA (AA genotype) individuals had 0.6% more MUFA than heterozygous alanine-valine (AV) (AV genotype) individuals and 1.7% more than VV (VV genotype) homozygotes (Taniguchi et al. 2004).

A similar relationship has been confirmed in cattle of the Fleckvieh breed (Barton et al. 2010). The meat of homozygous AA and heterozygous AV cattle had a larger share of MUFA than in homozygous VV individuals [least squares mean expressed as g (100 g)$^{-1}$ of all fatty acids; 42.9 for the AA and AV genotypes, 41.8 for the VV genotype; statistically significant difference at $P < 0.05$]. Furthermore, in the AA genotype, the content of tetradecenoic acid (C14 : 1 cis-9) was 20% higher than in the AV heterozygote and more than 37% higher than in the VV homozygote [AA (0.45); AV (0.36); VV (0.28); least squares mean expressed as g (100 g)$^{-1}$ of all fatty acids, statistically significant difference at $P < 0.05$] (Barton et al. 2010). The lipoprotein lipase (*LPL*) gene coding for lipoprotein lipase is regarded as a candidate gene associated with the lipid profile. Oh et al. (2003) reported the presence of three SNPs affecting the content of individual fatty acids. One was the c. 322*G* > *A* polymorphism, located in exon 2 of the *LPL* gene, which resulted in the *AA* genotype, characterised by a statistically significantly higher ratio of MUFA to SFA [*GG* (1.31); *GA* (1.36) and *AA* (1.42); $P < 0.05$)], significantly higher content of C18 : 3 *n*-3 acids [*GG* (0.33%), *GA* (0.36%) and *AA* (0.45%); $P < 0.05$] and a higher marbling score [*GG* (5.31); *AG* (5.49); *AA* (6.3); $P < 0.05$]. The inverse relationship was shown for C18 : 2 *n*-6 acids, with significantly higher content in homozygous *GG* individuals [*AA* (2.59%); *GG* (3.06%); $P < 0.05$]. A similar relationship was observed in the case of the c. 329*A* > *T* (exon 2) and c. 1591*G* > *A* (exon 9) polymorphisms, where specific alleles also influenced the lipid profile (Oh et al. 2013).

Conjugated linoleic acid is an important compound in terms of dietetic and health-promoting properties. Its health-promoting effect has been analysed with regard to the prevention of numerous disease entities, including arteriosclerosis and hypercholesterolaemia, immune system modulation, and antioxidant and anti-tumour activity (Yuan et al. 2014; Fuke and Nornberg 2017). Han et al. (2013) indicated a link between polymorphism G > A in exon 4 of the *LXR*α gene and the content of conjugated linoleic acid (least squares mean 0.595 for the *GG* genotype and 0.543 for the *GA* genotype, statistically significant difference at $P = 0.05$).

Unfavourable traits. For both breeding and processing, it is important not only to perpetuate economically beneficial alleles but also to eliminate those with a detrimental effect on meat quality. One of the best known examples of the negative effect of genetic polymorphism on meat quality parameters is a mutation in the ryanodine receptor 1 (*RYR1*) gene in pigs, which is involved in cellular calcium metabolism. The nucleotide substitution causes arginine to be replaced by cysteine, resulting in the development of a disease entity known as malignant hyperthermia (Fujii et al. 1991). In carriers of the unfavourable allele (heterozygous *CT* and recessive homozygous *TT* individuals), greater susceptibility to stress, aggressive behaviour, and inferior meat quality associated with reduced pH are observed (Van den Maagdenberg et al. 2008; Skrlep et al. 2010). The elevated body temperature and reduced meat quality of these animals are due to an increased release of calcium from the cell and accelerated glycogenolysis, causing a large amount of lactic acid to accumulate, which results in reduced pH and frequent occurrence of the pale, soft, exudative (PSE) meat defect (Laville et al. 2009). Molecular analyses can be used to reduce the occurrence of the unfavourable allele in the population (Barbut et al. 2008) and thus the amount of meat of poorer quality. Molecular research has also been successfully used to limit the occurrence of the autosomal recessive disease bovine leukocyte adhesion deficiency (BLAD), which causes significant losses for cattle breeders (Nagahata 2004).

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SNP DETECTION METHODS

Analyses of the effect of the molecular background on the qualitative characteristics of raw material of animal origin currently encompass numerous aspects from genetic research on SNPs, through whole-genome, proteomic and metabolomic analyses, to *in silico* studies revealing the properties obtained following peptide digestion or the activity of metabolic pathways. The varied research approaches are associated with a complex set of diverse research techniques.

Conventional polymerase chain reaction (PCR)-based methods. The basis for many molecular techniques is amplification of genetic material by polymerase chain reaction (PCR), in which the number of copies of a selected gene fragment is multiplied and then the resulting product is further analysed (Mullis et al. 1992). Based on PCR, methods involving the detection of SNPs have been created. This is achieved using restriction enzymes that recognise sequences specific to themselves and cut the DNA strand, in a method known as PCR-restriction fragment length polymorphism (PCR-RFLP). Polymorphism leads to the creation or loss of the unique restriction sites recognised by the enzyme and cleavage of amplified DNA sequence into fragments of various sizes. Digested amplicons are separated on agarose or polyacrylamide gels and it is detected which of the alleles is present in the test sample (Hashim and Al-Shuhaib 2019). However, to successfully apply the RFLP technique it is necessary to know the genomic context of the analysed sequence, as particular restriction enzymes are specific to particular polymorphic sites. Therefore, RFLP may be used to detection of previously confirmed SNPs but not to detection of new polymorphisms. Nevertheless, the RFLP technique is still very popular, mainly due to its relatively simple methodology and comparatively low cost (Bonilla et al. 2010; Anton et al. 2011). It is successfully used to detect numerous polymorphisms associated with meat quality (Taniguchi et al. 2004; Lan et al. 2007; Barton et al. 2010; Bonilla et al. 2010; Reardon et al. 2010). For example, the PCR-RFLP technique involving *Fok*I enzyme was used to analyse polymorphism in the growth hormone (*GH*) gene of pigs. The authors confirmed that animals with *GG* genotype had a lower body fat amount and higher muscle percentage in comparison with the animals carrying genotypes *AG* and *AA* (Bižienė et al. 2011).

Another method based on amplification and subsequent separation of a product by electrophoresis is PCR-single-strand conformation polymorphism (PCR-SSCP), which involves separation of a denatured PCR product through electrophoresis in polyacrylamide gel. The technique is based on the fact that polymorphism in the DNA sequence changes the mobility of DNA strands, which has been used to study polymorphism in the CAPN gene (Juszczuk-Kubiak et al. 2004). Other alternatives are allele-specific PCR (also performed using real-time PCR) (Vankan et al. 2010) and amplification refractory mutation system-PCR (ARMS-PCR) (Ribeca et al. 2013; Medrano and de Oliveira 2014). Both methods exploit the fact that amplification is inhibited when the 3'-terminal of the primer is not an exact match to the template. In this way, an appropriate primer design makes it possible to distinguish individual alleles on the basis of differences in the length of the PCR product. The ARMS method has been used in practice to detect missense mutations in the acyl-CoA dehydrogenase very long chain (*ACADVL*) gene, which is associated with growth traits in cattle (Zhang et al. 2015).

Real time-PCR and high resolution melting (HRM). There are also methods of SNP detection which are not followed by electrophoresis. Modifications of classical PCR, such as real-time PCR, facilitate analysis and expand research possibilities. The use of two probes labelled with different fluorescent dyes enables genotyping of samples without the need for separation by electrophoresis, which is essential in a classical PCR (Carrodeguas et al. 2005). An unquestionable advantage of real-time PCR is that this technique allows both detection and quantitative measurement of amplified products due to the use of standard curve (absolute quantification) or reference genes (relative quantification). The combination of reverse transcription and real-time PCR enables the analysis of differences in the expression of individual genes, such as those resulting from differences in the diet or rearing system (Ma et al. 2010). A real-time PCR-based method, used in the analysis of the molecular background of meat quality, is high resolution melting (HRM) analysis, which allows genotyping and discriminating of DNA sequence variants. HRM is an alternative method to PCR-RFLP and techniques involving probes such as TaqMan-probe-based real-time PCR. During HRM analysis, targeted DNA fragment is amplified in the presence of a fluorescent dye which binds to double stranded DNA. The proper analysis takes place after amplification when the products are gradually melted and the temperature increases, as a result, the dye is released and the fluorescence decreases, producing a characteristic melting profile. Profile of melting is measured

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to generate a characteristic curve, which reflects the mix of amplicons present in the sample. Melting profile is contingent on such factors as GC content, length of the sequence but also the presence of SNPs and small insertions and deletions (indels) which affect the shape of melting curve. Therefore, it is possible to distinguish genotypes carrying different polymorphic nucleotides. López-Rojas et al. (2017) confirmed that HRM is an efficient method to analyse SNPs associated with meat tenderness and the obtained results were consistent with those found by PCR-RFLP. Usefulness of HRM analysis was also revealed by Peng et al. (2013), who confirmed that HRM could be effectively used for genotyping of *GDF-8* gene.

Sequencing. PCR-RFLP and HRM reveal information about the presence or absence of polymorphisms. Sequencing is an even more precise method that can be used as it provides an entire nucleotide sequence. The Sanger method, in use since the 1970s, remains the most common sequencing method (Sanger et al. 1977). Unlike the RFLP and ARMS techniques, it provides information not only about a single polymorphism but also about the entire sequence flanked by the primers, enabling the precise detection of polymorphisms resulting from substitutions and of indel variations affecting single nucleotides. Due to their decreasing cost and increasing throughput, sequencing methods are increasingly included in routine analyses.

An integral element of sequencing studies is bioinformatic analysis of the data, which provides a more complete understanding of the context of the variation occurrence. The appearance of differences in the nucleotide sequence may entail changes in the amino acid sequence and in the structure and functionality of the protein, which can be detected during *in silico* analysis.

A limitation of the Sanger method is the relatively short length of fragment reads during a single analysis [usually 800–1 200 base pair (bp)], which is sufficient for the analysis of single polymorphisms or a few polymorphisms located close together, but not for the analysis of entire genes or of many polymorphic nucleotides situated in different parts of the genome. Such approach was applied in the analysis of polymorphisms in adenylosuccinate lyase (*ADSL*) gene. Mao et al. (2018) amplified and sequenced exons of *ADSL* to find polymorphisms associated with meat quality and carcass traits in domestic pigeons. Researchers found nine polymorphic nucleotides, and two of them (located in exons 10 and 11) displayed a significant association with a higher carcass rate. The Sanger method was also used in the analysis of the promoter region of protein kinase, AMP-activated, gamma 3 non-catalytic subunit (*PRKAG3*) gene. Ryan et al. (2012) investigated the relationship between SNP polymorphism, expression of gene and meat quality phenotypes in pork. The same method was applied by Zhou et al. (2021) to reveal three polymorphic nucleotides in the first exon of protein kinase, AMP-activated, gamma 1 non-catalytic subunit (*PRKAB1*) gene which were associated with the growth traits of goats.

SNaPshot. The throughput of classical sequencing by the Sanger method can be increased using the SnaPshot technique, which is currently used in forensic genetics, among other applications. The method is based on the extension of a strand by single nucleotides, called single-base extension (SBE), and makes it possible to analyse multiple polymorphisms in different parts of the genome during a single reading (Civáňová and Knoll 2007; Fondevila et al. 2017; Mehta et al. 2017). The method includes two multiplex PCR reactions (PCR with several primer pairs, enabling simultaneous amplification of many different genome fragments). During the first reaction, specific fragments flanked by the primers are amplified, and then the amplicons are purified using appropriate enzymes. During the second amplification, the primer, annealing just before the polymorphic nucleotide, is extended by another nucleotide, and at this stage dideoxynucleotides (ddNTP), labelled with various dyes are added, terminating the reaction (Mehta et al. 2017). The reaction products are separated by capillary electrophoresis in a genetic analyser. The use of ddNTPs labelled with various dyes makes it possible to distinguish individual alleles, and the varied length of the amplicons enables simultaneous analysis of multiple loci. For example, the SnaPshot method was used to study the relationship between polymorphism in the fatty acid-binding protein 4 (*FABP4*) gene and the content of unsaturated fatty acids and marbling in the meat of Hanwoo cattle (Oh et al. 2012) and the relationship between eight SNPs in five genes and meat traits such as instrumentally measured tenderness and sensory impressions (Gill et al. 2009).

CONCLUSION

There is no doubt that knowledge of the genetic basis of quality characteristics is a valuable tool in contemporary animal breeding that can be used to estimate the production potential of individual genotypes in the early stages of breeding work. Continually improving understanding of the genetic mechanisms underlying

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production traits allows breeders to influence the quality of raw meat in a more informed manner.

The increasing throughput and decreasing cost of molecular studies enable not only to detect single nucleotide differences but also to extend the analysis to entire genomes of animals in search of candidate genes associated with quality characteristics of economic significance. Such results are obtained for example by next-generation sequencing (NGS) methods. The high throughput of sequencing techniques and appropriate design of arrays enable simultaneous analysis of the associations between many traits. A study by Pegolo et al. (2019) based on an array of more than 20 000 SNPs showed a relationship between individual polymorphisms and traits such as carcass weight, meat colour, and WHC. The results obtained in whole-genome analyses form the basis for a search for potential molecular markers that can be further studied and verified using widely available methods, such as RFLP and Sanger sequencing, described above.

It should be borne in mind, however, that the final phenotypic effect is the result of multiple factors, not only genetic but environmental ones as well, such as diet or rearing system. Moreover, the genetic background itself is a complicated issue, because most traits are determined by multiple genes which often interact in complex ways. Nevertheless, advances in '-omics' technologies (genomics, proteomics, metabolomics, etc.), which integrate knowledge from the fields of biochemistry, molecular biology, physiology, and computer science, are providing a continuous insight into the complicated molecular system which is the basis for traits of importance for both animal breeding and food technology.

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