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Application of DNA markers for the assessment and characterization of genetic resources in sheep breeding

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Abstract

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This review summarizes the advances in molecular biotechnology for characterization of AnGR in sheep breeding. The review gives a brief summary on the development of genetic markers including both the classical genetic markers and more advanced DNA-based molecular markers – new generations of molecular markers for use in the genetic improvement of sheep breeds. This will help better understanding the characteristics of different genetic markers and the genetic diversity of sheep genetic resources.

In this review, we focus on the introduction of the most important DNA-based markers, and their various applications in characterizing sheep animal genetic resources (AnGR).

Keywords: Sheep animal genetic resources; (AnGR) Genetic markers; Molecular markers; DNA markers

Introduction

The development of every farm breeds has led to each having its own specific genetic characteristics that constitutes the genetic diversity of the population. These special animal genetic resources develop animal production for human food needs.

For evaluating the genetic structure and diversity of sheep populations various genetic markers are used. In previous studies morphological markers, biochemical markers, such as proteins and isozymes, were utilized.

With the rapid development of modern biotechnology, many different conventional DNA-based molecular markers had been used in sheep populations for assessment of genetic diversity – RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism) and SSR (Microsatellite DNA).

Nowadays novel strategies such as whole genome SNP chips and DNA barcoding have developed. The conventional and the latest DNA – based molecular markers assure much more precise and reliable information for the assessment of genetic diversity than previous markers.

Knowledge of the genetic diversity of livestock species and sheep in particular is important to maximize the potential of genome-wide association studies, and genomic prediction, conservation, management and use of Animal Genetic Resources (AnGR) (Fernández et al., 2011; Al-Mamun et al., 2015).

Genetic markers applied to AnGR assessments in sheep breeding

There are two main types of conventional genetic markers used to evaluate genetic diversity: morphological and molecular markers.

Morphological markers

Morphological markers refer to the external animal characteristics of sheep. They are used in the identification, classification, and characterization of genetic evolution of

different sheep populations (Gizaw et al., 2007). However the application of morphological markers is limited in the evaluation of quantitative traits. Only those traits that are controlled by a single locus can be used for morphological markers and their expression is reproducible, regardless of environmental conditions.

The molecular markers in turn are divided into:

1. Biochemical markers – variations in protein molecules;

2. Molecular (DNA markers) – related to differences in DNA structure.

Genetic markers are used to characterize:

1. Phenotypic diversity (phenotypic traits);

2. Chromosomal diversity (at the karyotype level);

3. Iimmunological diversity (blood groups, HLA antigens);

4. Protein diversity (isoenzymes);

5. DNA diversity of sheep populations (polymorphism of DNA sequences).

Modern trends in sheep breeding include the use of new molecular methods – DNA based markers on the application of DNA technologies, thus providing the industry being profitable and competitive.

Molecular markers (DNA based markers)

A molecular marker is based on the nucleotide sequence mutations within the individual's genome. They are the most reliable markers available. Molecular markers are used for evaluation of genetic variations at the DNA level between different populations and individuals that is taking place rapidly and directly. Molecular markers are developed quickly, and they are become more and more informative (Vignal et al., 2002; Liu & Cordes, 2004). Existing polymorphisms in the genome of organisms allow them to be used as markers to identify and distinguish representatives of the same species, as well as to breed and interspecific differentiation. Markers linked to a specific trait can be used to diagnose it in early stages, and based on its established localization on a specific chromosome to construct genetic maps (Albert et al., 1994; Stein et al., 1996). Marker's selection in small AnGR is an important trend in practical genetics (Marker Assisted Selection – MAS), suggesting the use of DNA markers associated with productivity traits trough genotyping and creation of genetic cards (Vignal et al., 2002).

RFLP (Restriction Fragment Length Polymorphism)

RFLP is a method based on the detection of different length fragments obtained by restriction of genomic DNA with a specific endonuclease enzyme. Genomic DNA restriction results in DNA fragments whose length and number may vary between species or breeds. The disadvantage of this technique is the need to use a large amount of DNA (5-10 μg), as well as the complicated procedurally and materially methodology.

In general, the RFLP technique is inferior to the current methods for genotyping of genetic resources like PCR (Polymerase Chain Reaction) because of the aforementioned considerations and because of the lower level of polymorphism than other DNA marker systems – SSR and AFLP. Despite of lower level of polymorphism compared to other DNA marker systems (SSR and AFLP) and the methodological limitations mentioned above, RFLP markers have been successfully applied in genetic resources research in animal husbandry. After first use to map the human genome (Botstein et al., 1980), they have been successfully applied in sheep (Montgomery et al., 1988).

Although their limited use, RFLP markers also serve as an important tool for evaluating genetic diversity within and across species (Old & Primose, 1998). Until 1995 RFLP markers have been identified for a total of 86 loci in the sheep genome (*Ovis aries*), 54 of them mapped to 16 different chromosomes (Montgomery et al., 1995).

Genetic diversity has been reported in Australian Merino sheep by Parsons et al. (1996) using 22 polymorphic marker lengths of restriction DNA fragments. Based on 6 microsatellites and 2 RFLP markers (associated with prolactin and β-lactoglobulin loci), Jandurovà et al. (2005) have studied the level of genetic variation in 3 indigenous sheep breeds from the Czech Republic and Slovakia.

In the last years many investigations have been done on polymorphism of different sheep genes and their relationship with productive traits using PCR –RFLP. A link between RFLP markers and some QTL in sheep has been reported by Mukberjee et al. (2006).

To analyze polymorphism in CAST and CPLG genes in sheep by PCR-RFLP method in Slovakia, G*ábor* et al. (2009) used 96 sheep of Tsigai, improved Valachian, East friesian, Lacaune breeds as well as the crossbreds Lacaune and Tsigai. In the total population of sheep, authors detected homozygous genotype MM – 0.87, heterozygous genotype MN – 0.13 for calpastatin gene. Homozygous genotype NN has not been observed. For the callipyge gene only homozygous genotype AA was identified. The heterozygous genotype AG and homozygous genotype GG were not detected.

The PCR-RFLP technique can be used to genotype individuals in cases where single nucleotide polymorphisms are localized in a restriction site, whereby after fragmentation by the respective enzyme specific fragments are generated, the

fractionation of which is most commonly performed electrophoretically (Vignal et al., 2002). Based on PCR-RFLP analysis, genetic diversity was characterized and genetic distances were established for different native sheep breeds from China (Xianglong et al., 2007)**;** Egypt (Abdel-Rahman et al., 2010); Indonesia (Prayitno et al., 2011) and others.

Candidate gene analysis with influence on sheep productivity traits and their subsequent application for accelerated selection – the so-called Marker-Assisted Selection (MAS) has been made by many authors in different countries.

Calpastatin Gene in Crossbreed Dalagh Sheep was investigated by Khederzadeh (2011), as well as in Zandi sheep (Khederzadeh et al., 2016) and in Zel sheep population of Iran (Gharahveysi et al., 2012).

Malewa et al. (2014) studied the growth hormone gene polymorphisms of Indonesia fat tailed sheep and their relationship with growth traits.

Cauveri et al. (2014) detected allelic polymorphism of exon 3 of leptin gene in Nilagiri sheep.

Subsequently, the analyzes of polymorphism in different genes of various breeds of sheep continued – like the analysis of Polymorphism of Caplstatin and Callipyge Genes in Saudi Sheep Breeds (Saleha, 2015), polymorphism of the *GDF9* Gene in Russian Sheep Breeds (Kolosov et al., 2015), polymorphism in DGAT1 Gene in Lori Sheep Breed (Nanekarani et al., 2016), genetic diversity of myostatin and calpastatin genes in Zandi sheep (Khederzadehet al., 2016), Toxoplasma gondii Type I, predominant genotype from sheep in South of Iran (Armand et al., 2017), genetic polymorphism in growth differentiation factor 9 (GDF9) gene related to fecundity in two Egyptian sheep breeds (El Fiky et al., 2017).

For evaluation of genetic variability which is an important element in conservation of genetic resources and breeding strategies a lot of studies have been done using RCR-RFLP markers in different sheep populations in Bulgaria.

Nakev et al. (2013) investigated the growth and development of skeletal muscle in connection with the expression of the myostatin gene (MSTN).

Georgieva et al. (2015) performed molecular analysis of ovine calpastatin (CAST) and myostatin (MSTN) genes in Synthetic Population Bulgarian Milk sheep.

Bozhilova-Sakova et al. (2016), and Dimitrova and Bozhilova – Sakova (2016) analysed the Ovine Myostatin (MSTN) and Callipyge (CLPG) in Karakachan sheep breed in Bulgaria. The same authors (2016) investigated the polymorphism in another gene – CAST Gene in Karakachan Sheep Breed. The last study showed absence of polymorphism in the Calpastatin gene in the studied population.

Dimitrova et al. (2016) investigated Ovine Myostatin (MSTN) in Northeast Merino sheep.

Bozhilova-Sakova & Dimitrova (2017), Dimitrova et al. (2017) through PCR-RFLP analysed three sheep genes CAST, MSTN and Callipyge genes in meat sheep breeds – Il de France sheep breed and Karnobat Merino sheep breeds.

Dimitrova et al. (2019) studied MSTN in Caucasian merino, Ascanian merino and reared in Bulgaria.

Bozhilova-Sakova et al. (2019) investigated the polymorphisms on ABCG2 and AA-NAT genes in different sheep breeds in Bulgaria.

Hristova et al. (2012) performed a review of the main the DNA markers and their application in animal breeding.

AFLP (Amplified Fragment Length Polymorphism)

AFLP technique first described by Vos et al. (1995) is one of the most powerful DNA fingerprinting techniques that could be used for genome studying. This method was used for molecular mapping, assessing genetic diversity, construction QTL maps, studying family relationship, assessing population genetic parameters and conservation of genetic pools in animals (Mueller et al., 1999). The AFLP technique provides an efficient marker system for revealing polymorphic loci and for linkage map construction.

Based on 16 EcoRI / TaqI primer combinations, Sangang et al. (2008) identified a total of 226 polymorphisms along the amplified fragments in 24 individuals of the thin-skinned Chinese Xinji sheep breed.

Because of the dominant type of inheritance, AFLP markers are not particularly suitable for the study of intra-breed diversity, but are highly informative in examining the relationships between different breeds of a species (Ajmone-Marsan et al., 2002), and those between closely related species (Buntjer et al., 2002). Despite some drawbacks, AFLP markers are widely used for genotyping and mapping of different sheep breeds (Fei et al., 2009; Anila et al., 2010; Mirhoseini et al., 2012).

Microsatellites (SSR – Simple Sequence Repeats)

Microsatellites are short (1-6 nucleotides), repeating monomeric sequences of type (AT) n, (GC) n, (ATT) n, etc. Di- and tetranucleotide repeats occur predominantly in the non-coding regions, whereas 57% of the trinucleotide repeats occur in regions in or around genes. Their use as DNA markers is facilitated by their codominant nature and by a quick and easy method of detection.

For the detection of microsatellite alleles a fragment analysis by capillary electrophoresis on automatic sequencer is used. In this case, multiplex PCR is possible. The method allows the analysis of several loci in one gel at a time, with minimal amounts of sample DNA (10-100 ng) for analysis, and allelic lengths can be determined with a high degree of accuracy (Adam-Blondon et al., 2004).

The advantages of microsatellite markers over RAPD markers are expressed both in their significantly higher reliability in terms of stability and repeatability of the results obtained, as well as in the higher level of polymorphism and codominant type of inheritance.

In recent years, a number of studies have demonstrated the close location of some microsatellite loci with candidate genes in terms of quantitative traits or so-called *Quantitative Trait Loci (QTL)*. Two microsatellite markers – BM1329 and OarAE101 are associated with the (closely localized to) Fecundity (FecB) gene localized on chromosome 6 in the sheep genome, have been used to indirectly identify this gene in different sheep breeds (Weimann et al., 2001; Nowak and Charon, 2001).

Microsatellites have been extensively used as DNA markers in genetic studies in various sheep breeds (Buchanan et al., 1994; Parsons et al., 1996; Canon et al., 2001; Altarayrah et al., 2007; Bozzi et al., 2009; Ceccobelli et al., 2009; Tolone et al., 2012).

SSRs markers were applied in sheep breeding in relation to animal paternity testing, defining the genetic structure of populations, assessing genetic diversity within and between breeds, and establishing of phylogenetic relationships (MacHugh et al., 1994; Ritz et al., 2000). These types of marker systems have also been successfully implemented for genetic mapping and QTL mapping (Hiendleder et al., 2003; Kühn et al., 2003). Over 1000 microsatellite markers have been mapped in sheep (Maddox et al., 2001).

Microsatellite markers are of greatest use in assessment of genetic diversity in sheep. Founded in 1989 by Weber May, microsatellite known as Short Tandem Repeats (STRs) or Simple Sequence Length Polymorphisms (SSLPs) in recent years are the most popular markers for genetic research in livestock. SSR Analysis is the "Gold" Standard Method for Studying Sheep Breeding Genetic Diversity (Tolone et al., 2012; Sheriff and Alemayehu, 2018).

Kusza et al. (2008) performed a comparative study of 41 sheep populations from 8 Central and Eastern European countries, including 5 Bulgarian sheep breeds: 2 Tsigai sheep populations (Rhodopes and Stara Planina Tsigai), 2 Marishki (Byala and Vakla), Pleven Black-headed. In the study, the genetic structure and diversity of the breeds was estimated on the basis of 16 microsatellite markers, and based on the dendrograms concerning the Bulgarian breeds, the authors conclude on the genetic similarity between the two Tsigaysk and respectively between the Marishka sheep populations and the Pleven Black-headed breed population identified as genetically unique based on SSR markers.

Hristova et al. (2014; 2017) reported the microsatellite based genetic diversity and population structure of seven Bulgarian indigenous sheep breeds (Breznishka, Sofiiska, Copper-Red Shumenska, Karakachanska, Local Karnobatska, Blackhead Plevenska and Starozagorska). The greatest distance (0.643) was found between the populations Local Karnobatska and Starozagorska, while the smallest one (0.108), between the Copper-Red Shumenska and Karakachanska. The constructed phylogenetic tree based on Neighbour-Joining method separates the investigated sheep breeds into two main clusters: one including Blackhead Plevenska Breznishka and Local Karnobatska, and the other one consisting of the four remaining breeds – Copper-Red Shumenska, Karakachanska, Sofiiska and Starozagorska sheep.

A number of studies have been conducted in another European countries such as Spain (Dies-Tascon et al., 2000; Arranz et al., 2001; Alvarez et al., 2004; Rendo et al., 2004; Legaz et al., 2008); Slovakia (Kusza et al., 2009); Italy (Pariset et al., 2003; d'Angelo et al., 2009); Switzerland (Stahlberger-Saitbekova et al., 2001); Austria (Baumung et al., 2006); Baltic Republics – Lithuania and Estonia (Tapio et al., 2005a); Northern Europe (Tapio et al.,005b).

Similar studies have been conducted in the Balkan countries – Greece (Ligda et al., 2009); Serbia (Cinculov et al., 2008a); Slovenia and Croatia (Ivancovic et al., 2005); Albania and Kosovo (Hoda et al., 2009). Population structure and genetic diversity studies in sheep based on SSR markers have also been conducted in multinational studies, such as Peter et al. (2007) covering 57 breeds of sheep from 15 different countries (12 European and 3 from the Middle East – Egypt, Saudi Arabia and Turkey), by Handley et al. (2007) – 29 breeds from Greece, Croatia, Italy, Spain, France, Romania, Hungary, Czech Republic, Germany, etc. of Kusza et al. (2011), with a range of 13 breeds from Croatia, Serbia, Romania and Turkey.

SNP (Single Nucleotide Polymorphism)

Single Nucleotide Polymorphisms represent the most common DNA markers in the human genome. One SNP is observed on average once every 1000 sun (Brookes, 1999). According to Brookes (1999), SNPs represent single base positions in genomic DNA in which different alternative nucleotide sequences (alleles) exist. All SNP Genotyping Techniques Denaturing Gradient Gel Electrophoresis (DGGE),

Single Strand Conformation Polymorphism (SSCP), Cut Length Fragment Polymorphisms, Cleavage Fragmentation Analysis Heteroduplex Analysis (HA), Denaturing High Performance Liquid Chromatography (DHPLC), Direct Sequencing, and more have been successfully applied to characterization of genetic resources in animal husbandry, the prior information on the sequence of the respective polymorphic sites being essential (http://cgil.uoguelph.ca/QTL/SNPs.htm).

SSCP has been successfully used as a method for the study of genetic diversity in milk protein and growth hormone genes in native Portuguese sheep to create a breeding program based on marker selection (Bastos et al., 2001)

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New sequencing methods are much more effective in identifying genetic variation within large populations (Todorovska et al., 2010).

Of the listed methods for detecting new SNP tags, the most used and accurate, though expensive and time consuming method is the Direct Sequencing. Compared to traditional Sanger-based sequencing methods, DNA sequencing by synthesis (SBS technology) is emerging to be the next generation of DNA sequencing, much more efficient and with significant genetic representation research (Fuller et al., 2009).

Several studies have investigated accuracy of genotype imputation and its impact on the accuracy of genomic selection in dairy and beef cattle through the adoption of high-density SNP panels, and more recently, whole-sequence data (Roberts, 2007; Su et al., 2008; Erbe, 2012; Hayes et al., 2012; Ventura et al., 2014; Dodds et al., 2014; Bolormaa et al., 2015; Ventura et al., 2016; Brito et al., 2017). Heaton et al. (2014) identify parentage SNPs for use in globally diverse breeds and to develop a subset for use in North American sheep. Starting with genotypes of 2915 sheep and 74 breed groups provided by the International Sheep Genomics Consortium (ISGC), they analyzed 47,693 autosomal SNPs by multiple criteria and selected 163 with desirable properties for parentage testing. On average, each of the 163 SNPs was highly informative (MAF≥0.3) in 48±5 breed groups.

An effective and novel procedure for genotyping SNPs is DNA Microarray technology, which can screen more than 40 000 sequences in a given genome at a time (Lipshutz et al., 1998). By wrapping the SNP50 Beadchip, comprising about 50 000 SNP markers, Kijas et al. (2009; 2012) genotyped 2819 sheep specialized in different directions belonging to 74 breeds from different continents (Asia, Africa, North and South America, Europe and Australia). The results obtained in this study reveal SNP polymorphism in over 90% of the studied loci, which is an indication of the high level of genetic diversity in these sheep breeds compared to other breeds of animals. African and Asian populations clustered separately from breeds of European origin sampled from Australia, New Zealand, Europe, and North America. The identification of a SNP subset is able to assign individuals into broad groupings demonstrates even a small panel of markers may be suitable for applications such as traceability.

Mitochondrial DNA (mtDNA) as a DNA marker

The mitochondrial genome shows a high degree of conservatism in various mammals. Due to the fact that mtDNA is inherited from the maternal lineage, and due to the lack of recombination processes between different genes in the mitochondrial genome, most studies are devoted to establishing the origin and tracing the phylogenetic relationships between closely related species and between populations of the same species (Bruford et al., 2003). In one of the first studies in sheep based on mtDNA polymorphism, three distinct lines were reported – haplotype groups A, B and C.

Significant similarities in mitochondrial sequences have been found between the European mouflon (*Ovis ammon musimon*) and one of these haplotype groups (B) in domestic sheep (Hiendleder et al., 1998, 2002). An extensive study on 48 different sheep breeds from the regions of Europe, Caucasus and Central Asia was conducted by Tapio et al. (2006). This study identified 4 haplogroups of sheep in the Caucasus region (A, B, C, and D), 3 in Central Asia (A, B, and C) and 2 in Europe (A and B), and the results confirm historical assumptions for the migration of some breeds of domestic sheep from the Middle East through Russia into northern Europe.

The use of mtDNA polymorphisms as genetic markers for genetic diversity studies is limited. Based on a study of variability in the "D-loop" region of the mitochondrial genome, by the PCR-RFLP method, Xiaglong et al. (2007) found low level of genetic diversity (0.0421) in 9 Chinese aboriginal sheep breeds. Compared to nuclear DNA markers, the potential for error in the study of the genetic diversity of populations is much greater when using mtDNA as a genetic marker. This is explained by the reported, although contradictory, data on mtDNA inheritance other than maternal and paternal lineages (Zhao et al., 2004), as well as the likelihood of recombination processes, both intramolecular and between the genomes of individual mitochondrial lines (Piganeau et al., 2004).

There are several sheep breeds , which were recently characterized using mtDNA in Albania, Italia and Greece

(Hoda et al., 2010; 2014) and mtDNA in other countries (Cinkulov et al., 2008; Pariset et al., 2011; Dotsev et al., 2019; Ganbold et al., 2019).

Conclusion

These briefly review showed the role of DNA markers in different areas of sheep breeding – paternity testing, biodiversity and conservation of sheep AnGR. Integration of information from all sources along with a search for direct markers and finding their causative sites for the QTL is required.

The new era of *omics* technology provides beneficial processing as well as analysis and integration of a large amount of data. Thus *omics* technology will provide valuable information regarding the precision of selection of appropriate molecular markers according to the goals of molecular investigation of sheep AnGR and include it in the Marker Genome Selection in the near future.

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