

# Identification of Molecular Markers Specific to the Bovine Species Used in the Production of Food Products of Animal Origin

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## Abstract

Molecular markers are a helpful tool for species detection of meat origin, in order to determine with accuracy the possible fraud of animal-based food products. In this study, blood, milk and cheese samples were taken from the Romanian “Bălțata” bovine species. In the blood extraction stage, the commercial Wizard Genomic DNA Extraction Kit was used, and for the milk and cheese extraction, the SureFood@PREP Advanced kit was used. Target DNA amplification in all the three matrices was performed with the StockMarks for Cattle kit. It was found that the analyzed loci had the size of the fragments within the reference range given by the kit used, which concludes that the blood, milk and the cheese have the same origin, from the same animal of the bovine species. Therefore, through the genotyping technique, traceability of food products can be achieved and the species of origin can be identified.

**Keywords:** DNA markers, genotyping, traceability, PCR Multiplex, bovine

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## Introduction

The use of molecular markers is a novelty in the management of genetic resources, with implications upon the effect of selection and shortening of the span between the generations in animals of economic interest (Pop et al., 2018). The main utility of molecular markers is represented by their use in various diagnosis or therapy techniques, an increase of the specificity and accuracy of obtained results leading to the generation of the safest and most real data possible (Kelemen et al. 2018). DNA markers

have developed exponentially starting with the discovery of the Polymerase Chain Reaction (PCR) technique, have been modified throughout time and improved continuously, thus leading to the present multitude of types of DNA markers, all based on the PCR technique (Zăuleț et al., 2008, Mocan et al., 2018). PCR is used in many research fields—biological, clinical, forensic, and diagnostic—and has revolutionized research in life sciences (Călinoiu and Vodnar, 2018; Vodnar and Socaciu, 2014; Călinoiu et al., 2019a; Călinoiu et al., 2019b; Stana et al., 2016; Vasile et al., 2017;

Vodnar et al., 2010; Călinoiu et al., 2016; Mitrea et al., 2017, Călinoiu and Vodnar, 2020; Teleky and Vodnar, 2019; Martău et al., 2019; Popescu et al., 2016, Szabo et al., 2019; Precup et al., 2017).

Generally, a DNA marker is based on a DNA sequence located anywhere on the genome chromosomes, provided that the region where it is found includes a detectable variation that makes it possible to differentiate between two or more related individuals (Zăuleț et al., 2008). The genetic information of living organisms is coded into DNA, with the configuration consisting of four types of nucleotides (A, G, C, and T) which form the letters of the genetic code. The genetic code presents different repetitive genetic sequences, and individuals have various repetitive motives transmitted from parents to descendants. These sequences are located in regions that do not code for proteins and can be of different types: mini-satellites, microsatellites, LINE sequences, SINE sequences, etc. Of all these, it is the microsatellites that are of the most interest in the performance of DNA-based tests. They are spread throughout the entire genome, are highly polymorphic and transmitted from genitors to descendants according to the Mendelian laws of heredity. The polymorphism of microsatellites is reflective of the genetic inheritance and allows the detection of differences between various individuals. Microsatellites can be easily amplified by the PCR (Polymerase Chain Reaction) reaction and afterward characterized by means of an automated genetic analyzer (Zăuleț et al., 2008). Therefore, DNA markers are extremely useful for the establishment of the identity of individuals, to verify paternity, traceability of food products and, not the least, to determine the species origin of meat, for an accurate establishment of potential frauds of products of animal origin (Zăuleț et al., 2008).

The difference, on sequence levels, between the satellite sequences and elements dispersed from the genome of bovines cannot be performed because both types of sequences contain in their structure common structural units (Barendse et al., 1994). This DNA sequence is never found individually, but only in the duplicated form (in this case, named Bov-A2) or associated with 73 pb tRNA<sup>Gly</sup> pseudogene (in this case, being named Bov-tA). SINE BOV-A2 AND BOV-TA elements represent approximately 1.6% of the bovine

genome (Barendse et al., 1994). Eukaryotes can include, at a particular time, RNA molecules that are reverse-transcribed and afterward integrated into chromosomes (Rogers, 1985; Weiner et al., 1986). This class of elements includes the repetitive sequences SINE, LINE, and processed pseudogenes. In most circumstances, SINE elements are derived from ARNt molecules, except for Alu sequences from primates and B1 sequences from rodents (Okada et al., 1995). By contrast to the SINE elements, which are up to 400 pb long, the LINE sequences have lengths of up to several kilo-pairs of bases (Scott et al., 1987). The mammalian genomes have a very high number of such sequences, the human genome including approximately 100,000 (Hwu et al., 1986).

Besides Bov-B and other LINE sequences have been discovered in the bovine genome: BATPS, located in the second intron of the gene that codes the ATP synthase (Dyer et al., 1989). These repetitive elements end in a variable number of tandem repetitions named microsatellites (Tautz et al., 1984). Microsatellites are short repetitive sequences, which include a variable number of repetitions of a unit of 1 – 5 pb. The most numerous such repetitions are poly (A), followed by poly (TG) or poly (CA). Due to their high variability, the latter types of repetitions are extremely important DNA markers. Microsatellites including TG or CA repetitions have been used intensely for the creation of genetics maps in bovines and other mammals. Besides their high level of polymorphism, these microsatellites have another technical advantage: they can be easily amplified through the PCR reaction, the results were reproducible. It is estimated that approximately 45% of the bovine macrosatellites are associated with SINE elements (Kaukinen et al., 1992; Vaiman et al., 1997). This high level of association between the microsatellites and the SINE elements seems to be characteristic of the *Bovidae family*. Molecular markers are a helpful tool for species detection of meat origin, in order to determine with accuracy the possible fraud of food products of animal origin.

The purpose of this study was to use the molecular markers for bovine detection of meat origin, in order to determine with accuracy the possible fraud of the targeted food products (milk, cheese). The method used to determine the molecular markers was the Multiplex PCR

molecular biology technique, which consists of traceability of a food product, from animal to finished product. Therefore, blood, milk and cheese samples were taken from a bovine from the Romanian "Bălțata" species. In the blood extraction stage, the commercial Wizard Genomic DNA Extraction Kit was used, and for the milk and cheese extraction, the SureFood®PREP Advanced kit was used. Target DNA amplification in all the three matrixes was performed with the StockMarks for Cattle kit. The principle of the method consisted of determining the genetic profile of the 3 samples taken in the study and analyzing each specific locus of this species.

## **Materials and methods**

### **Materials**

In order to optimize the test, we studied the blood from the Romanian "Bălțata" bovine species, the milk collected in one day from multiple animals and the cheese obtained from this milk. We performed a genetic finger-printing of the animal, milk and end product (obtained from the milk originating from multiple animals), being able to obtain traceability from the animal to the finished product. Samples have been collected in special, vacuum tanks, with EDTA as an anticoagulant agent.

### **Reagents**

Wizard Genomic DNA Purification Kit from Promega, UV/VIS Lambda 25 spectrophotometer (Perkin Elmer), Horizontal electrophoresis machine (BioRad), StockMarks for Cattle Kit (Applied Biosystems), 11 pairs of primers with fluorescent marking, StockMarks PCR Buffer, dNTP mixture, DNA control, Ampli Taq Gold Polymerase, Genescan – 500 ROX Size Standard, Thermocycler GeneAmp 9700 (Applied Biosystems), ABI Prism 310 automatic genetic analyzer (Applied Biosystem), and all the other reagents have been procured from Applied Biosystem and Sigma.

### **Extraction and purification of DNA from blood**

The isolation of genomic DNA was performed with the Wizard Genomic DNA Extraction Kit, in accordance with the methodology and instructions provided by the manufacturer. Therefore, the kit included a four-phase saline extraction process. The first phase consisted of the lysis of red cells, followed by the lysis of white cells and their nuclei in a nuclear lysis solution. RNA was digested

by incubation with RNase (ribonuclease). The following phase included the precipitation of proteins with a concentrated saline solution while genomic DNA with higher molecular mass remained in the solution. In the end, genomic DNA was concentrated and desalified by precipitation with isopropanol.

The extraction and purification of the DNA from the blood was done using the Wizard Genomic DNA Extraction Kit, following the methodology and instructions provided by the manufacturer. The blood collected on EDTA was gently shaken, then mixed with 900 ml of cell lysis solution and incubated for 10 minutes at room temperature. During incubation, it was shaken 2-3 times for complete cell lysis. After centrifugation (14000 g, 20 seconds, at room temperature), the supernatant was removed, and 300 ml of nuclear lysate solution was added over the cell suspension and stirring was performed by successive pipetting. If agglomerations of cells occurred, the incubation at 37 °C was applied until their disappearance. Then 100ml of protein precipitation solution was added to the nuclear lysate and vortexed for 10-20 seconds. Centrifugation took place for 3 min. at 14000 g and room temperature and the protein precipitate appeared as brown sediment. The supernatant was transferred to 300 ml of 100% isopropanol and stirred until the DNA appeared as slivers, then centrifuged for 1 min. at 14000 g. The supernatant was discarded and 1 ml of ethanol was added to the precipitate, followed by centrifugation at 14000 g, 1 min. The supernatant was removed by decanting. The precipitate was dried for 10-15 min. and rehydrated in a minimum volume of water, 50-100 ml, overnight at 4 °C. The DNA solution was kept at 2-8 °C. The purity and concentration of DNA were determined spectrophotometrically (DO 260/280nm).

### **DNA extraction from milk and milk product (fresh cheese)**

Isolation of genomic DNA for these types of matrixes was performed with the SureFood®PREP Advanced kit, manufactured by Congen, Germany, according to the methodology and instructions provided by the manufacturer. Therefore, the preparation of the samples was done by preheating the elution buffer at 65 °C, and cell lysis by the addition of 400 µl lysis buffer, 20 µl proteinase K, over the sample to be analyzed. The mixture was vortexed at maximum speed, then

**Table 1.** Bovine-specific loci amplified with the StockMarks kit

Locus	Pigment	Colour	Fragment size (pb)
TGLA227	FAM	Blue	64-115
BM2113	FAM	Blue	116-146
TGLA53	FAM	Blue	147-197
ETH10	FAM	Blue	198-234
SPS115	FAM	Blue	235-265
TGLA126	JOE	Green	104-131
TGLA122	JOE	Green	134-193
INRA23	JOE	Green	193-235
ETH3	NED	Yellow	90-135
ETH225	NED	Yellow	240-270
BM1824	NED	Yellow	170-218

**Table 2.** Components and volumes of the PCR mixture for the 11-plex reaction

Components	Volume (µl)
StockMarks PCR Buffer	3
dNTP mix	4
AmpliTaq Gold DNA Polymerase	0.5
Primer mixture	5.5
Deionized water	1
Total volume	14

**Table 3.** PCR reaction protocol

PCR machine	Reaction tube	Times and incubation errors						
		Initial phase	31 cycles			Final extension	Final phase	
			Denaturing	Anelation	Extension			
GeneAmp 9700	GeneAmp Reaction Tube 0.2-ml	10 min. 95°C 1 cycle	45 seconds 94°C	45 sec. 61°C 30 seconds of the ramp	1 minute 72°C 30 seconds of the ramp	60 minutes 72°C 1 cycle	120 minutes 25°C 1 cycle	∞ 4°C

incubated at 65 °C for 30 min. at 1000 rpm. The tubes removed from the incubator are centrifuged for 1 minute at 12.000 rpm, then the mixture was filtered. DNA purification was accomplished by successively pipetting, three times, 550 µl of wash buffer. The DNA was dried by centrifugation at 12.000 rpm for 2 min. And finally, DNA elution occurred by pipetting the elution liquid (100 µl) and centrifuging at 12.000 rpm for 3 min. The DNA tube was kept at 4 °C until genetic analysis was performed.

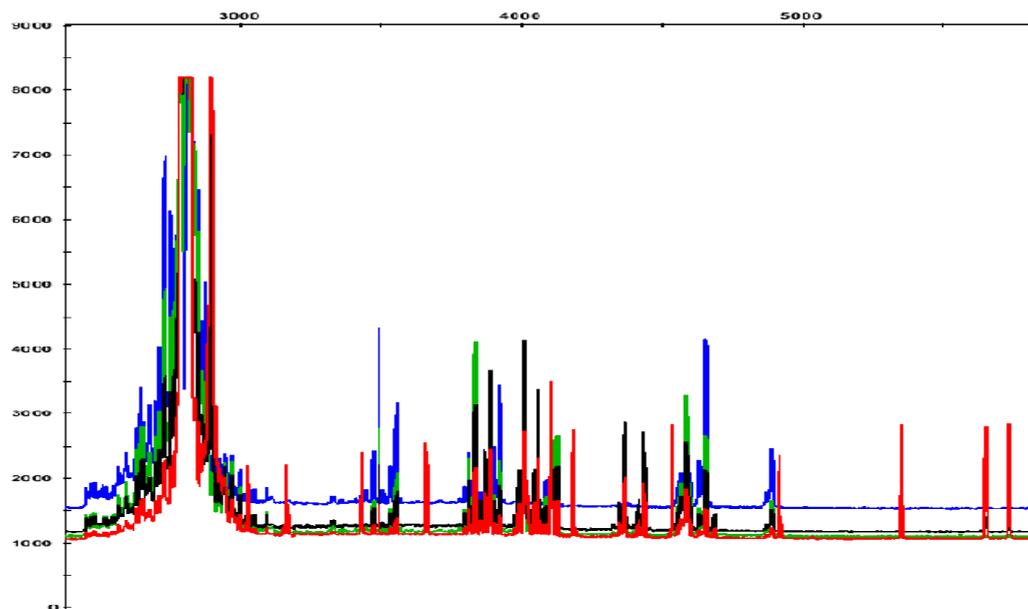
**DNA amplification**

The amplification of microsatellites from extracted DNA samples was performed with the StockMarks kit. The kit included 11 pairs of primers necessary for the amplification of 11

microsatellites (Table 1). The pairs of primers were marked with three fluorescent pigments (FAM, JOE, and NED) so that all 11 microsatellites can be analyzed in a single pass through the ABI Prism 310 automatic genetic analyzer.

Preparation of the 11-plex reaction: Reagents involved in the reaction (Table 2) were dispensed in a tube of 0.5 ml and vortexed; 14 µl of the reaction mixture was added in a tube of PCR for each tested animal; 11 µl of isolated DNA from each animal to be tested, were added after diluted accordingly; vortexing and centrifuging; the tubes were inserted in the PCR machine.

For each tested genotype, a single multiplex PCR reaction was performed considering the low risk of unspecific amplification, protocol given in



**Figure 1.** Unprocessed genetic profile for a bovine specimen

Table 3 below. Following the completion of the PCR reaction, the obtained products were combined with formamide and molecular mass standard ROX – 500 and inserted in a single tube for each tested animal.

Following the performance of the PCR reaction, samples are prepared for the detection of amplified fragments. In the first phase the amplification products of the PCR reaction were diluted with a volume of 90  $\mu$ l deionized water; In the next step, the samples to be loaded into the equipment were prepared accordingly: Gene-Scan-500 ROX Size Standard (molecular weight marker), deionized formamide and diluted amplification products; Immediately before introducing the samples into the equipment, they were denatured by heating at 95  $^{\circ}$ C and then abruptly cooling on ice; Finally, the samples were introduced into the ABI Prism 310 automatic genetic analyzer. The amplified fragments from each sample were subjected to capillary electrophoresis, followed by fluorescence detection using a special LASER reader. The resulting profiles were done using the GeneScan Analysis and Genotyper (Applied Biosystems) programs. Following the analysis, the genetic profiles of each specimen were obtained.

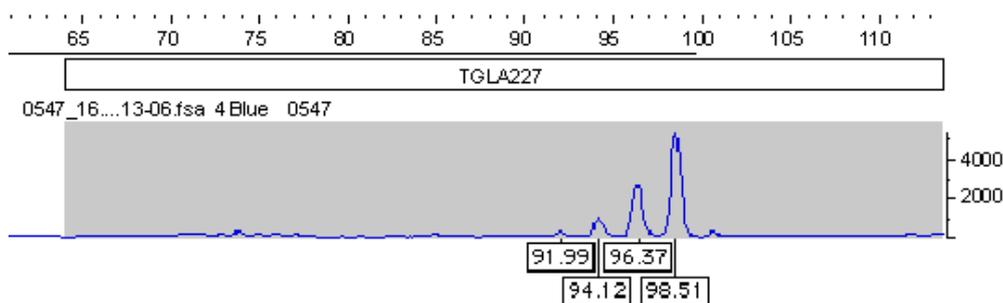
### Results and discussions

Figure 1 includes an unprocessed genetic profile resulting from the migration and separa-

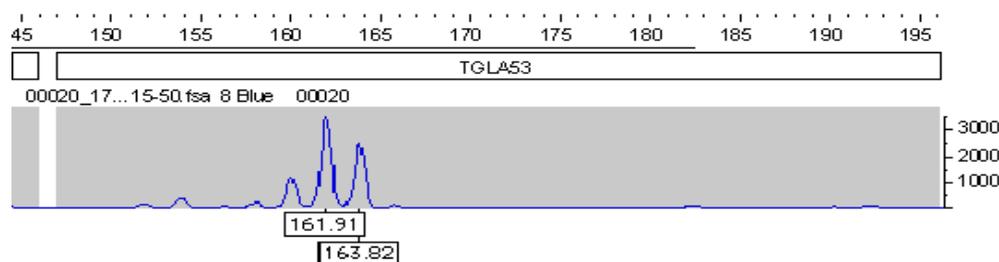
tion of amplification products for a single specimen. Fragments marked red represented the molecular mass marker and those marked in blue, green and black were amplified microsatellites of the respective specimen. The raw profile provided following the detection of amplified fragments was processed with computing software, obtaining the genetic print of the analyzed individual.

In order to be able to interpret the results correctly, consideration must be given to multiple details. The primers in the StockMarks kit performed the amplification of microsatellites that presented a varied number of dinucleotide repeats. They provided a characteristic appearance to the obtained genetic profiles.

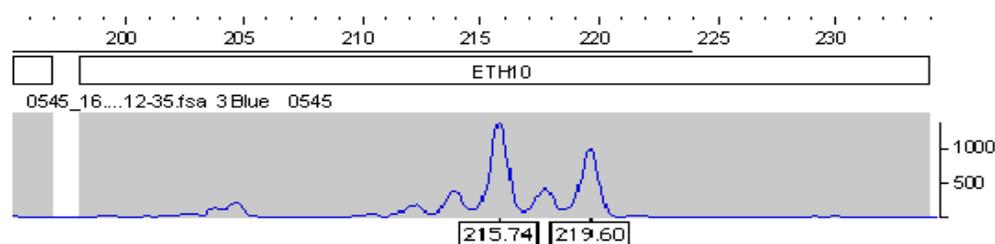
It must also be specified that the number of alleles present on the particular locus depended on the homo- or heterozygosity of the analyzed individual. Thus, homozygous individuals had a single allele on a particular locus, therefore the electropherogram indicated only one signal. Heterozygous individuals had two different alleles on a particular locus, therefore the electropherogram indicated two signals. The shape of obtained signals also depended on the size of the amplified microsatellites. Thus, we can state that the profile performed with DNA markers was unique for each individual and, thus, it was the most effective method of individual identification.



**Figure 2.** Profile characteristic to a homozygous individual in an analyzed locus



**Figure 3.** Profile characteristic to a heterozygous individual with alleles different by two pairs of bases



**Figure 4.** Profile characteristic to a heterozygous individual with alleles different by four pairs of bases

The specific profile of a homozygous individual included, besides the main signal, a train of signals of increasingly less intensity (figure 2). The three smaller signals that occurred besides the main signal were due to the fact that the microsatellite amplified presented dinucleotide repeats. They had a size in pairs of decreasing bases of every second pair: 98.51 pb the main signal and 96.37 pb, 94.12 and 92 pb the other train signals.

In the case of heterozygous individuals with alleles different by 2 pb, a characteristic signal similar to the one presented in individual 00020 on locus TGLA53 (figure 3) was obtained.

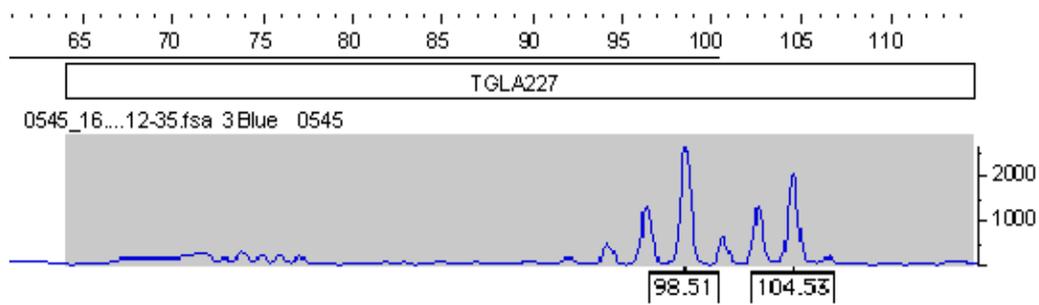
For heterozygous individuals with alleles different by 4, respectively 6 pb, signals similar to those in figures 4 and 5 were obtained. All these things provided a characteristic appearance

to genetic profiles obtained for each analyzed specimen.

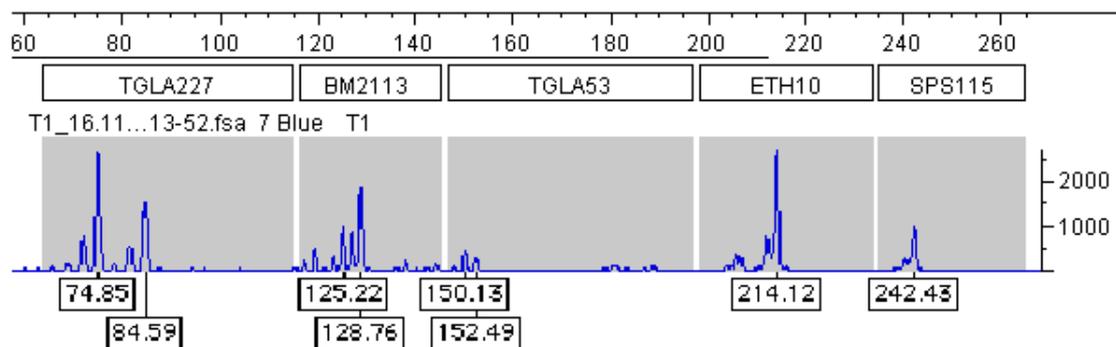
Figures 6, 7 and 8 included an example of the genetic profile of a specimen analyzed with the StockMarks kit.

Molecular markers can also be used for the estimation of genetic variability in populations, as well as in the study of relationships between populations. The genetic variation can be established depending on the percentage of heterozygosity (H) or, in case of establishment of the population diversity, as a real number of alleles ( $A_e$ ).

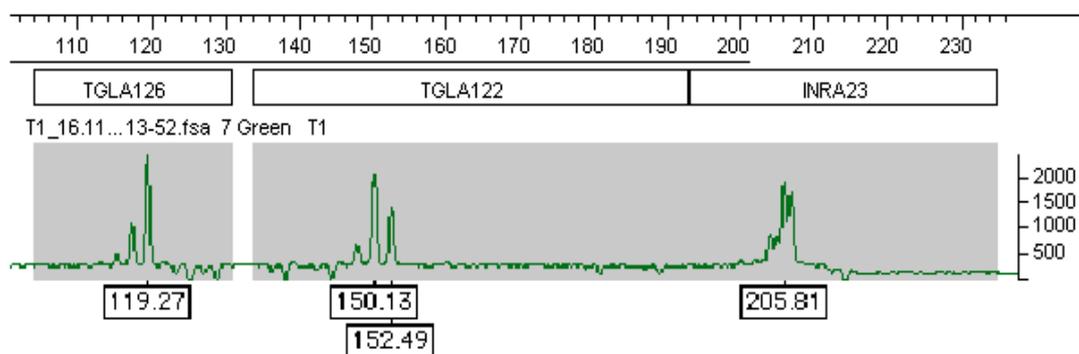
In most situations, in the studies regarding the genetic diversity of cattle, it was preferred to use loci that were known as polymorphic. This outlined the fact that genetic variability



**Figure 5.** Profile characteristic to a heterozygous individual with alleles different by six pairs of bases



**Figure 6.** Genetic profile of a bovine specimen for loci TGLA227, BM2113, TGLA53, ETH10, and SPS115

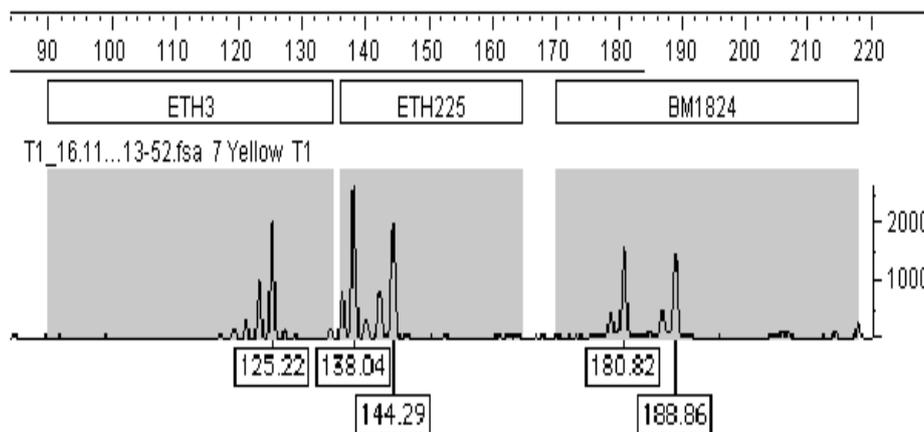


**Figure 7.** Genetic profile of a bovine specimen for loci TGLA126, TGLA122, and INRA23

estimated in the case of domestic species was not comparable to the variability of wild populations. Comprehensive studies on the genetic variation of different races of cattle have been performed initially starting from the loci that code serum proteins or which determined the polymorphism of blood types. Variability was expressed in percentages of heterozygosity, which represented a number of alleles per locus and effectiveness in genetic traceability tests.

Recent studies that use DNA markers have been employed. They are much more relevant and can provide the researchers with more complete information with regard to the analyzed populations.

Following the performance of the genotyping study, by means of the analysis of electropherograms, for the three matrices (blood, milk, and cheese) traceability had been established (animal – food products). Table 4 and figures 9,



**Figure 8.** Genetic profiles of a bovine specimen for loci ETH3, ETH225, and BM1824

**Table 4.** Dimensions, in base pairs, for each locus, on each matrix

Sample/ Locus	TGLA227 1	TGLA227 2	BM2113 1	BM2113 2	TGLA53 1	TGLA53 2	ETH10 1	ETH10 2
P1 - blood	78.94	90.12	133.44	138.28	163.23	-	200.91	215.82
P2 - milk	78.88	90.09	133.56	138.28	163.24	-	201.03	215.97
P3 - cheese	78.77	90.03	133.55	138.28	163.27	-	201.03	215.91
Sample/ Locus	SPS115 1	SPS115 2	TGLA126 1	TGLA126 2	TGLA122 1	TGLA122 2	INRA23 1	INRA23 2
P1 - blood	242.34	250.10	113.30	-	130.28	-	200.91	212.36
P2 - milk	243.31	250.10	113.25	-	130.28	-	201.03	212.50
P3 - cheese	242.35	250.16	113.27	-	130.20	-	201.01	212.43
Sample/ Locus	ETH3 1	ETH3 2	ETH225 1	ETH225 2	BM1824 1	BM1824 2		
P1 - blood	113.20	-	142.81	146.02	180.67	182.02		
P2 - milk	113.25	-	141.09	146.02	180.77	182.81		
P3 - cheese	113.27	-	141.94	146.01	180.75	182.80		

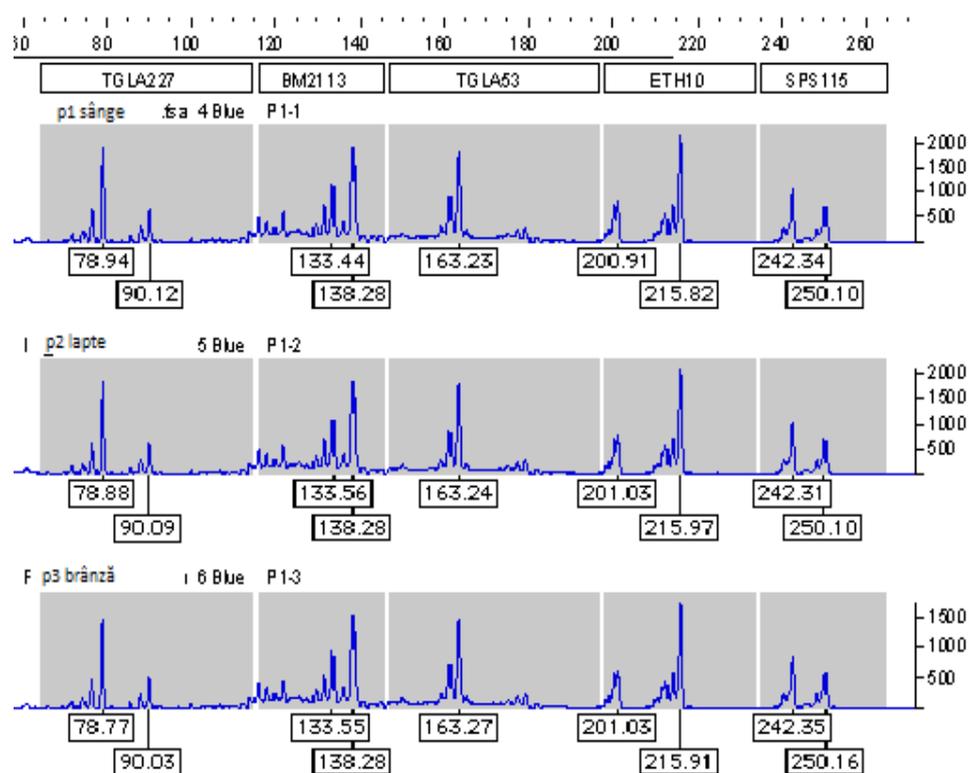
10 and 11 included the results obtained for each locus (expressed in base pairs)

Because of the integration of cattle in the human food chain, there are frequent circumstances that involve the DNA analysis of bovines (*Bos taurus*), in order to establish potential food fraud (substitution of beef with inferior meat, respectively horse meat). However, scientific publications that reported the content of information with regard to the STR loci (Short Tandem Repeat loci) analysis are relatively rare and this because the analysis method was expensive and required state-of-the-art equipment. In 2009, Van de Goor et al. performed studies to establish traceability analyzing 16 STR-loci. The number of loci used for the genotyping of STR for bovines was sufficient for the analysis of animal - food product traceability. Moreover, the specificity of loci

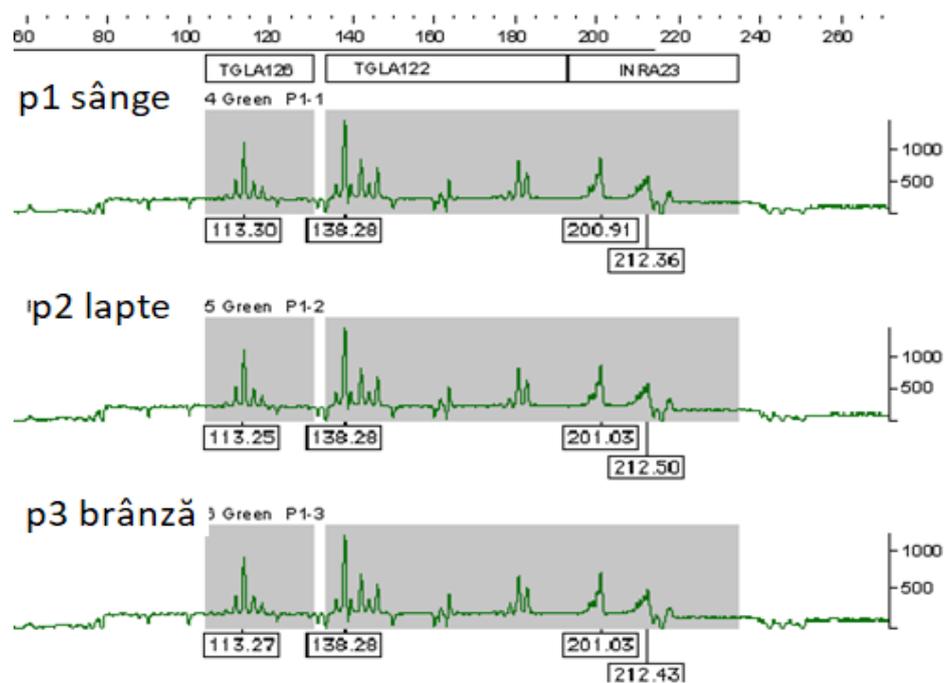
(characterized by base pairs) had been assessed in the review of traceability based on the genotype data. In 2011, Putnova et al. performed studies for the identification of traceability and parental control performed by cattle farmers in the Czech Republic by means of the analysis of 17 STR loci.

In 2017, Xu et al. proposed a study with published results on the well-established correlation between the size of alleles and the variation of allele dimension, these analyses identified that the values of STR-loci, given in pairs of base pairs can be used to characterize traceability.

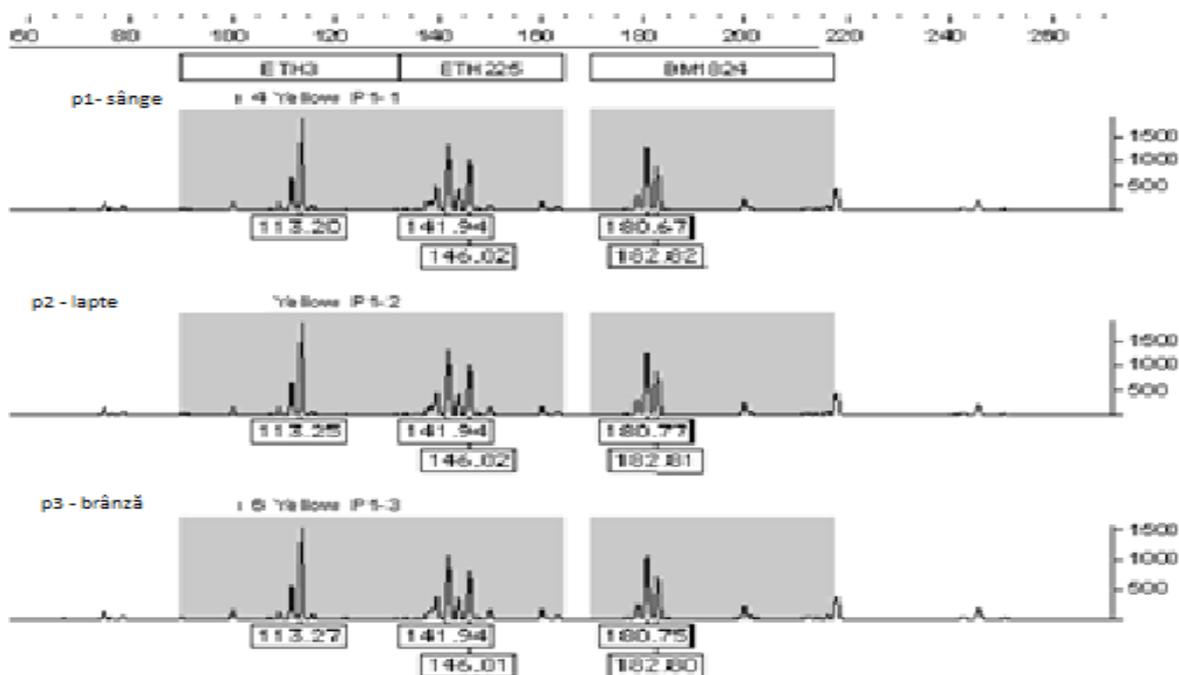
Considering the existing studies, our study included the analysis of 11 STR loci, recommended by the International Society for Animals Genetics (ISAG) for the routine use in the test and identification of parental and traceability for the



**Figure 9.** The genetic profile obtained on blood, milk and cheese samples for loci TGLA227, BM2113, TGLA53, ETH10, and SPS115.



**Figure 10.** The genetic profile obtained on blood, milk and cheese samples for loci TGLA126, TGLA122, and INRA23.



**Figure 11.** The genetic profile obtained on blood, milk and cheese samples for loci ETH3, ETH225, and BM1824

bovine species (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824).

### Conclusions

For each genotype tested, a single PCR Multiplex reaction was performed to amplify the specificity of blood samples, milk, and cheese, respectively, from the bovine species. After amplification, the fragments were subjected to capillary electrophoresis for their size detection, completed by obtaining genetic profiles on each sample taken in the study.

The results obtained after the genotyping process shown that the 3 samples fall within the reference values given by the kit used for the bovine species. Thus, it can be concluded that the 3 studied samples (blood, milk, and cheese) came from the same studied bovine species, which showed that traceability of the final product can be achieved, from animal to product.

The practical utility of this method consists in the performance of traceability of a food product - animal, primary product (milk), and product (cheese) - by the procurement of the genetic print in order to complete the herdbooks. Thus, genetic printing can be performed for each individual on a

farm, and this print can be accessed subsequently from a database in order to establish traceability to an end food product.

This is a novelty in the field of food safety, namely that if, following the production of end products, the animal shows signs of illness which have not been discovered when preparing the products, they can be withdrawn from the market selectively, only particular products that originate from a particular farm, from a particular animal.

DNA markers are extremely useful for the establishment of the identity of individuals, to verify paternity, traceability of food products and, not the least, to determine the species origin of meat, for an accurate establishment of potential frauds of products of animal origin.

DNA markers developed in this study can help identify the species of fresh, cooked and heat-treated chicken, beef and pork, as it is a simple, economical and quick identification process compared to other methods.

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