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Molecular markers used in genetic characterization of Romanian Black Spotted Cattle

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Introduction

During the last three decades, major advances have been made in mammalian genetics. New methods have been developed and applied to investigating the genetics of the cattle and to improve its performance. Animals with superior traits, such as high milk production and lean carcasses in the case of cattle, or speed and strength in the case of horses, are used as breeding stock for subsequent generations. The developments in molecular genetics allowing the detection of genes responsible for economic traits and genetic disorders have opened a new area in farm animal selection.

The classical method of selection is based on physical observation of inherited traits in adult animals and the careful maintenance of lineage records by breeding organizations.

The DNA profile test is more powerful than conventional methods because it detects DNA-sequence information that is highly variable. The work performed using microsatellite markers has shown that they are valuable tools for examining genetic diversity and phylogeny in many species. This technology provides a sensitive method for parentage verification, individual identification and the estimation of relatedness.

α-lactalbumin (α-LA) is a major milk protein essential for the biosynthesis of lactose at the level of mammary glands. β 1,4-Galactosyltransferase 1 transfers galactose from UDP-Gal to *N*-acetylglucosamine, which constitutes its normal galactosyltransferase activity. At the level of the epithelial cells of the mammary glands, in the presence of α-LA, it transfers Gal to Glc, which is its lactose synthase activity. The lactose released into the milk together with α-LA dehydrates the milk through osmosis, this being a condition for its quality. α-LA directly influences the quality and the volume of the milk since it is directly involved in the lactose synthesis (Ashwell et al., 1997).

K-casein (k-CN) and β-lactoglobulin (β-LG) are two of the most important proteins in the mammals' milk synthesized by the epithelial cells of the mammary glands. They play a crucial role in the milk quality and coagulation process and are essential for the fabrication of cheese and butter. Certain genetically variants of β-LG are linked with the presence of a high percentage of casein and milk fat.

Bovine citrullinemia is an inherited disorder of the urea cycle that has been found in Holstein-Friesian cattle (Citek et al., 2004). This disorder results from a deficiency of the enzyme argininosuccinate synthetase, which catalyzes the conversion of citrulline to argininosuccinate. The mutation responsible for this disorder has been characterized as a single-base substitution (C-T), converting the CGA codon that codes for arginine-86 to

TGA, a translation–termination codon (Padeeri et al., 1999). This conversion results in a restriction site elimination and in a truncated peptide product (85 amino acids long rather than the normal 412 amino acids) that lacks enzymatic activity (Healy et al., 1990; Viana et al., 1998).

Bovine Leukocyte Adhesion Deficiency (BLAD) is a lethal autosomal recessive disease in Holstein cattle characterized by a greatly reduced level of expression of the β2 heterodimeric integrin. The molecular basis of BLAD is a single point mutation (A-G) at position 383 in the cDNA of the CD18 gene. This mutation results in a substitution of a glycine for an aspartic acid at position 128 in the D128G protein (Gerardi et al., 1996; Jorgensen et al., 1993; Meylan et al., 1997; Rutten et al., 1996; Shuster et al., 1992). DNA restriction with endonuclease *TaqI* or *HaeIII* could detect differences between healthy and affected calves by elimination of the enzyme restriction site.

Deficiency of Uridine – 5´Monophosphate Synthase (DUMPS) is a genetic disorder, which interferes with pyrimidine biosynthesis and is inherited as a single, two-allele, autosomal locus (Kuhn et al., 1994; Shanks et al., 1992). The enzyme uridine – 5´-monophosphate (UMP) synthase catalyses the conversion of orotic acid to UMP, the precursor of all other pyrimidine nucleotides and a normal constituent in the milk of cows and other ruminants (Shanks et al., 1990; Shanks et al., 1989).

Material and Methods

DNA extraction

Blood samples for DNA genotyping were obtained from ICDB Baloteşti farm. Genomic DNA isolation was performed with Wizard Genomic DNA Extraction Kit (Promega) from fresh blood sample (300 µl). The total amount of isolated DNA was resuspended in sterile TAE and after spectophotometric quantification diluted to 50 ng for each reaction.

Microsatelitte genotyping

Genetic characterization of Romanian Black Spotted population was made using eleven microsatellites (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824). They were chosen based upon the polymorphism detected in other breeds (Fries *et a*l. 1993; Barendse *et a*l. 1994; Bishop *et a*l. 1994).

Amplification of the STR loci was realized by multiplex PCR using StockMarks for Cattle Paternity PCR Typing Kit (AppliedBiosystems). Thirty-one amplification cycles were performed in GeneAmp® PCR System 9700: 94ºC/45 sec; 61°C/45 sec; 72°C/60 sec. The first denaturation step was performed at 95ºC/15 min and the last extension was 72°C/60 min.

PCR – RFLP genotyping

For BLAD, citrullinemia, DUMPS, β-LG and k-CN genotyping we performed a simple polymerase chain reaction (GeneAmp® PCR System 9700) followed by enzymatic restriction. Genomic DNA was amplified in a 25µl reaction containing: PCR buffer, $MgCl_2$, dNTPs, AmpliTaq DNA Polymerase and nuclease-free water, sense and antisense primers, for 45 cycles for each gene type using different protocols (Table 1).

Table 1: Primer sequences and annealing temperatures.

	Primer sequences	Protocol
Citrullinemia	for.5'-GGGCCAAAAAGGTGTTCATTGAGGACATC-3' rev.5'-CAAGTATGTGTCTCACGGCGCCACAGGAA-3'	95°C/30 sec; 57°C/30 sec; 72°C/1 min.
BLAD	for. 5'-CCTTCCGGAGGGCCAAGGGCT-3' rev. 5'-CTCGGTGATGCCATTGAGGGC-3'	95°C/30 sec; 57°C/30 sec; 72°C/1 min.
DUMPS	for. 5'-GCAAATGGCTGAAGAACATTCTG-3' rev. 5'-GCTTCTAACTGAACTCCTCGAGT-3'	95°C/30 sec; 58°C/30 sec; 72°C/1 min.
α-lactalbumin	for. 5'-CTCTTCCTGGATGTAAGGCTT-3' rev. 5'-AGCCTGGGTGGCATGGAATA-3'	95°C/30 sec; 58°C/30 sec; 72°C/1 min.
β-lactoglobulin	for. 5'-GTCCTTGTGCTGGACACCGACTACA-3' rev. 5'-CCCAGGACACCGGCTCCCGGTATAT-3'	95°C/30 sec; 57°C/30 sec; 72°C/1 min.
k-casein	for. 5'-ATCATTTATGGCCATTCCACCAAAG-3' rev. 5'-GCCCATTTCGCCTTCTCTGTAACAGA-3'	95°C/30 sec; 57°C/30 sec; 72°C/1 min.

The first denaturation step was performed at 95ºC (10 min) and the last extension was 30 minutes (72°C).

PCR products were digested with restriction endonucleases (Tables 2a, 2b) at 37°C for 3 h. Restricted products were analyzed by electrophoresis in 2% Agarose High Resolution gel stained with ethidium bromide.

Table 2a: Restriction enzymes used to digest the PCR products.

Length of PCR products bp	**Restriction enzymes**	**Normal homozygote** restriction fragments bp	**Affected homozygote** restriction fragments bp	**Carrier heterozygote** restriction fragments bp	**References**
Citrullinemia 200	*Ava II*	112/88	200	200/112/88	Padeeri et al., 1999
BLAD 136	*Taq I*	108/28	136	136/108/28	Viana et al., 1998 Ribeiro et al., 2000
DUMPS 108	*Ava I*	51/36/21	87/21	87/51/36/21	Shanks and Robinson, 1990

Table 2b: Restriction enzymes used to digest the PCR products.

Length of PCR products bp	**Restriction enzymes**	**AA genotype** restriction fragments bp	**BB genotype** restriction fragments bp	**AB genotype** restriction fragments bp	**References**
β-LG 262	*Hae III*	153/109	109/79	153/109/79	Medrano et al., 1990
k-CN 350	*Hinf I*	134/84	266/84	266/134/84	Medrano et al., 1990
α -LA 166	*Mnl I*	78/52/36	114/52	114/78/52/36	Bleck and Bremel, 1993 Mao, 1994

PCR Amplification and Sequencing for α-LA

The most used method to distinguish between AA, BB and AB genotypes of *α-LA* gene is to cut the 166 bp PCR products (obtained using primers in Table 1) with *Mnl I* endonuclease at the recognition site GAGGNNNNNN↓. The lengths of the fragments we must obtain are 78/52/36 bp for AA, 114/52 bp for BB and 114/78/52/36 bp for AB (data not shown because of some problems with the restriction enzyme). To confirm the *α-LA*

genotypes we decided to perform sequencing. The amplified fragments were purified with the Wizard PCR Preps DNA Purification System Kit (Promega) and amplified for sequencing using the ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction and were run on ABI Prism 310 Genetic Analyzer. DNA Sequencing Analysis 5.1 Software (AppliedBiosytems) was used to process the sequences which were aligned with the Clustal X multiple alignment program and refined manually.

Results and Discussions

One of the difficulties in implementing a selective breeding program in cattle stocks is maintaining pedigree information. Another difficulty in managing a selective breeding program is the loss of genetic variability and increase in inbreeding. The effects of inbreeding in cattle will result in a decrease in genetic variability, which will limit the potential for genetic gain from artificial selection.

In our study, we have analyzed 100 specimens of Romanian Black Spotted cows from ICDB Baloteşti farm. Successful amplification yields allele peaks with the associated PCR stutter bands within a maximum range of eight base pairs from the allele peak. For this StockMarks Kit, all eleven loci are dinucleotide repeats. Size calling is based on ABI 500 ROX Size Standard and was done by base pairs size ordering, from smallest to largest. The number of allele peaks depends on whether the individual tested is a heterozygote or homozygote. Allele frequencies, the observed heterozygosity *(H_o)* and expected heterozygosity *(H_e)* were calculated using the software program Cervus 2.0 (Marshall *et al*., 1998) and the results are present in Table 3.

The size range of alleles at the individual loci varied between 75 and 252 bp. High level of heterozygosity was observed in Romanian Black Spotted cows population studied for BM1824, INRA23 and ETH10 loci and a lower level for the TGLA53 locus.

For TGLA53 locus we observed a significant deviation from the Hardy-Weinberg equilibrium. This might be caused by the population substructure (hybridisation between subspecies), selection acting on linked loci, and a null allele segregating in the population or a sex-linked locus. It is possible that the deviation from Hardy-Weinberg equilibrium across this locus shows that the population is genetically subdivided. Perhaps two or more populations have recently been mixed within the Romanian Black Spotted breed.

Table 3: Statistical analysis for Romanian Black Spotted cattle.

Locus	**Alelle size** (bp)	**H_o**	**H_e**	**PIC**	**HW**
TGLA227	75-104	0.745	0.794	0.762	NS
BM2113	120-141	0.638	0.843	0.814	NS
TGLA53	148-183	0.362	0.684	0.647	*
ETH10	210-222	0.750	0.776	0.740	NS
SPS115	242-252	0.513	0.564	0.518	NS
TGLA126	116-126	0.617	0.677	0.612	NS
TGLA122	138-172	0.681	0.779	0.739	NS
INRA23	197-213	0.766	0.832	0.805	NS
ETH3	113-130	0.702	0.717	0.684	NS
ETH225	134-152	0.745	0.820	0.786	NA
BM1824	179-192	0.787	0.760	0.707	NA

***, **, *** - significant deviation, NS - non significance, NA - not done; H_o**=observed heterozygosity; **H_e**=expected heterozygosity; **PIC** -polymorphic information content; **HW=**Hardy-Weinberg equilibrium test

The second part of our article is dedicated to the three diseases genotyping. We studied the genes responsible for citrulinemia, BLAD and DUMPS.

With primers we designed (Table 1), normal homozygote genotype for citrullinemia exhibits two bands of 112 and 88 bp, carrier heterozygote shows three bands of 200, 112 and 88 bp, and affected homozygote only one band of 200 bp. In our case all, the studied specimens were normal. After digestion with *Ava II* endonuclease, two fragments of 112 and 88 bp characteristics were detected (Fig. 1).

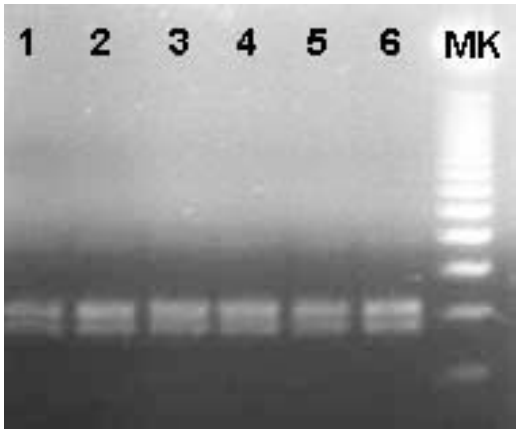

Figure 1. Electrophoresis pattern of citrullinemia locus after digestion with *Ava II* enzyme.
Line 1-6, two fragments of 112 and 88 bp indicate homozygous cattle with normal alleles. Line 7, molecular size marker (50 bp DNA Step Ladder).

The length of PCR products obtained using specific primers for BLAD is 136 bp (Table 1). After digestion with *Taq I* endonuclease normal BLAD genotype have shown two bands of 108 and 28bp, carrier heterozygote three bands of 136, 108, 28bp and affected homozygote only one band of 136 bp. In our study only two fragments of 108 and 28bp, characteristics for the normal cattle were detected (Fig. 2A).

DUMPS carriers are phenotypically normal, but have only half the normal activity of uridine monophosphate synthase (Harden and Robinson, 1987). Also, during lactation, carriers excrete an elevated level of orotic acid in milk and urine (Robinson et al., 1984).

The primers we used have provided an amplification fragment of 108 bp. Normal homozygote for DUMPS revealed three bands of 51, 36 and 21 bp, carrier heterozygote four bands of 87, 51, 36 and 21 bp and affected homozygote only two bands of 87 and 21bp.

In Figure 2B a typical result of DUMPS genotyping is shown. After digestion with *Ava I* endonuclease three fragments of 51, 36 and 21bp, characteristics for the normal cattle were detected. Our results indicate that the population of Romanian Black Spotted dairy cattle studied is free from DUMPS. Because of the economical significance of the DUMPS mutation and its recessive mode of inheritance, attention has to be paid to any case of a male having in his origin any known DUMPS carrier.

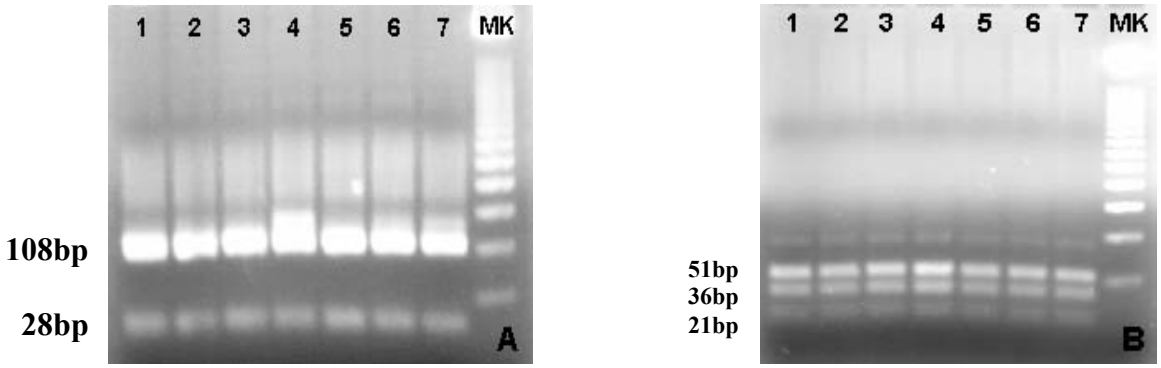

Figure 2. Electrophoresis pattern of amplified fragments after digestion with *Taq I* enzyme (BLAD) and *Ava I* (DUMPS) enzyme.
A. *Lines 1-7, two fragments of 108 and 28bp characteristic for homozygous cattle (BLAD).*
B. *Lines 1-7, three fragments of 51, 36 and 21bp characteristic for homozygous cattle (DUMPS).*
Line 8, molecular size marker (50bp DNA Step Ladder).

The last part of our paper is designated to genotypes for β-LG, k-CN and α-LA identification for the Romanian Black Spotted cattle.

Eleven genetic variants which encode different forms of the β-LG protein have been discovered, thus influencing the quality of the milk: A, B, C, D, E, F, G, H, I, J and W. Out of these, the A and B forms are of the most interest since they are associated with milk production performances, its quality and processing. The BB homozygote individuals supply milk rich in fat and protein, very valuable in the process of cheese making, while the AA homozygote ones supply milk with a low percentage of fat but in a larger quantity.

In our study, detection of the genetic polymorphism of the bovine β-LG locus has been done by digestion of the PCR fragment of 262 bp with *Hae III* endonuclease (Medrano and Aguilar-Cordova, 1990). The enzymes cut the PCR products in two fragments of 153 and 109bp for the AA genotype and in two fragments of 109 and 79 bp for the BB genotype. Heterozygotes AB are a combination of the two alleles A and B (three fragments of 153, 109 and 79bp) (Fig. 3A).

k-CN influences the coagulation process of milk and its content of protein. Up to now, 9 genetic variants which encode different forms of k-CN: A, B, C, E, F, G, H, I and J have been discovered. Out of these, only the A and B alleles are more frequent in bovine populations. Studies made so far prove that milk containing the B form of k-CN has much higher lactodynamographic properties than those containing the A form.

Analysis of the genetic polymorphism bovine k-CN locus has been done by digestion of 350 bp PCR products with *Hinf I* endonuclease (Medrano, and Aguilar-Cordova, 1990). Length of restriction fragments is 134/84 bp for AA genotype, 266/84 for BB genotype and 266/134/84 bp for AB genotype. In the population studied, we have revealed only AA homozygous cattle (Fig. 3 B).

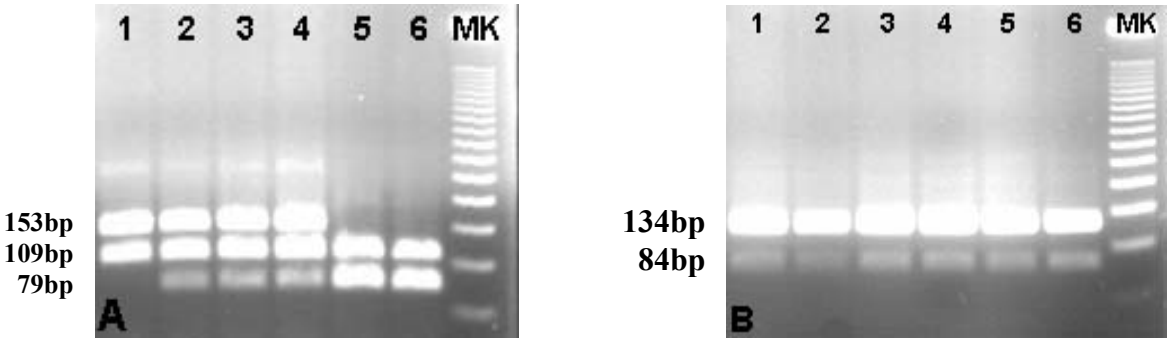

Figure 3. Electrophoresis pattern of amplified fragments after digestion with *Hae III* enzyme (β-LG) and *Hinf I* enzyme (k-CN).
A. *Line 1, two fragments of 153 and 109bp characteristic for AA homozygous cattle; lines 2-4, three fragments of 153, 109, 79bp characteristic for AB heterozygous cattle; lines 5 and 6, two fragments of 109 and 79bp characteristics for BB homozygous cattle (β-LG).*
B. *Lines 1-6, two fragments of 134 and 84bp characteristic for AA homozygous cattle (k-CN).*
Line 7, molecular size marker (50bp DNA Step Ladder).

The milk-specific protein α-LA is a key part of the lactose synthase complex in mammary epithelial cells. This enzyme complex is responsible for the production of lactose, the major osmole in milk and a major determinant of milk volume. Through the sequencing method mentioned above, one hundred Romanian Black Spotted cattle were genotyped and the different variants (AA, BB and AB) were analyzed for any potential effect on milk production traits (data not shown).

In Figure 4, a region of a PCR fragment of the sequenced α-LA gene is shown. Restriction site for *Mnl I* (left) normally used to detect different α-LA genotypes was identified .

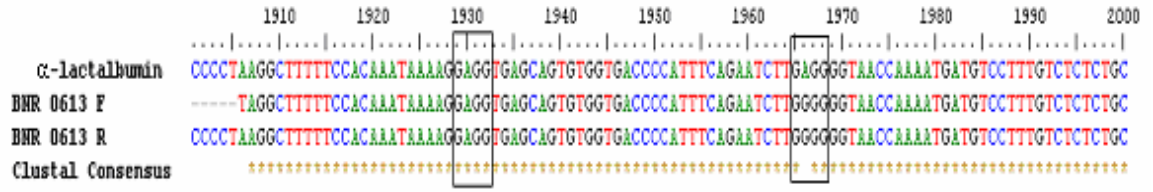

Figure 4. Clustal X alignment exemplification of an amplified product containing restriction sites of α-LA gene (BB genotype).

Conclusions

Microsatellite markers are more likely to detect small differences between populations than other methods due to their high levels of allelic variation, being able to discriminate in both overall heterozygosity and mean number of alleles. Our data demonstrate an important level of polymorphism detectable with microsatellite loci within the Romanian Black Spotted cattle population. In cattle breeding this technology has the potential to be of great use in monitoring levels of genetic variation within stocks as well as for parentage and relatedness purposes.

The importance of polymorphism of milk proteins in the selection of dairy cows has been shown, since milk protein genetic variants have a significant effect on the composition and properties of the milk. In cattle breeding, the genetic diseases produce significant effects that lead to a decrease in or loss of performance.

Detection methods based on PCR amplification and RFLP analysis are powerful tools to detect the presence of polymorphism of gene coding for α-LA, β-LG, k-CN and responsible for some genetic disorders (DUMPS, BLAD, Cittrulinemia).

Identified genotypes for α-LA, β-LG and k-CN genes for Romanian Black Spotted cattle confirm that the breed is a dairy one. For all the three genetic diseases evaluated our results point out that all tested cattle are normal, displaying normal genotypes.

Using the PCR-RFLP technique, we established for the first time in Romania, an easy and efficient method that can be use to determine the genotype of dairy cattle and to correctly identify the carrier and the affected animals for these genetic disorders.

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