DIFFERENTIATION OF MANGALITZA PIGS FROM LARGE WHITE AND WILD BOAR IN BIOLOGICAL SAMPLES AND MEAT PRODUCTS USING DNA MARKERS

A.S. Ardelean Costin[1*], V.A. Bâlteanu[1], M. Mihaiu[1]

[1]University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, Calea Mănăştur 3-5, 400372 Cluj-Napoca, Romania

Abstract

The demand for Mangalitza derived meat products on the market increased significantly in the last years. Although their number increased in the last decades, this is not enough to satisfy market demand for high quality pork products. In this context, fraudulent practices, which might consist of meat substitution or undeclared crosses with more productive pig breeds or wild boar, might occur. DNA-based methods might offer a viable solution to limit these possible fraudulent practices. In this respect, we tested the possibility to use some single nucleotide polymorphisms (SNP) located in SLC45A2 and MC1R genes as DNA markers for differentiation of Mangalitza from Large White pigs and from wild boar in biological samples and in some pork products. The genotyping data revealed that a combination of these two markers might be successfully used for this purpose.

Key words: *Mangalitza pigs, wild boar, pork products, authenticity, DNA markers*

INTRODUCTION

In the last 50 years, Mangalitza breed and its three coloured varieties (red, blonde and swallow-belly) almost disappeared in Romania and Hungary due to competition with more productive commercial pig breeds (e.g. Large White, Duroc etc) or crossbreeding [1]. However, in the last twenty years Mangalitza population in both countries grew significantly due to an increased demand on the European and American markets for cured-ham and other pork products obtained from this traditional breed. In this context, possible fraudulent practices, which might consist of meat substitution or undeclared crosses with commercial pigs (e.g. Large White), might occur. Furthermore, undeclared crossing of Mangalitza with wild boar increased in the last years in Romania, in order to obtain game-like meat.

Using a genome wide SNP genotyping based approach, Mangalitza coloured varieties, which in Hungary are registered as distinct breeds [2], can be clearly distinguished from some commercial pig breeds [1]. However, this method requires a specialized and expensive equipment. Therefore, the availability of simple and low-cost DNA-based methods is essential to certify the authenticity of Mangalitza derived pork products.

Simple tandem repeats (STR) or single-nucleotide polymorphisms (SNP) were used for differentiation of some pig breeds or from wild boar [3, 4, 5, 6]. *MC1R* is a major gene associated with pigmentation in pigs [7]. Different allelic variants (E^+, E^{D1}, E^{D2}, E^P and e) found in this locus were successfully used to differentiate several pig breeds from wild boar [8]. However, Mangalitza pigs and wild boar carry the wild type *MC1R* E^+ allele [9], therefore they cannot be differentiated only using this marker.

In a previous study, we reported for the first time that in Mangalitza pig red *versus*

*Corresponding author: costinalexandrasilvia@yahoo.com

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blond pigmentation is strongly associated with missense SNPs located in *SLC45A2* gene [9].

Therefore, the objective of the current study was to test if some single nucleotide polymorphisms found in *SLC45A2* and *MC1R* genes, involved in pigmentation, could be used as genetic markers to differentiate Mangalitza from Large White pigs and Romanian wild boar.

MATERIAL AND METHODS

Samples and pork products tested

In order to carry out the planned genetic analyses, reference pig blood samples from Mangalitza breed individuals, red (RedMaRo, n=30) and blonde (BloMaRo, n=18) varieties (Figure 2) and Large White (LaW, n=10) were collected on K_3EDTA tubes.

Fig. 1. Individuals of the Mangalitza pig breed, red and blonde colour varieties

Romanian wild boar (WbRo) muscles samples (n=20) were collected during organized hunting events in the season.

In addition, some commercial pork products were collected for testing:

- pork products (producer P1), produced from Large White pigs: smoked meat, bacon and sausage (Figure 2a);
- pork products (producer P2), produced from Mangalitza pigs, red variety: smoked meat, bacon and sausage (Figure 2b);
- pork products (producer P3), declared as being produced from Mangalitza pigs: fresh meat, bacon and sausage (Figure 2c).

DNA analysis procedure

DNA extraction was performed from 200 µl of blood with the ReliaPrep™ Blood gDNA kit, using a protocol recommended by the producer (Promega, USA). In the case of meat samples, prior to column purification procedure, few milligrams of tissue were transferred to Eppendorf tubes and lysed with 200 µl lysis buffer and 20 µl proteinase K. After a short centrifugation for 1 min at 10000 g, the supernatant from each sample containing DNA was transferred in new tubes and purified using the same kit.

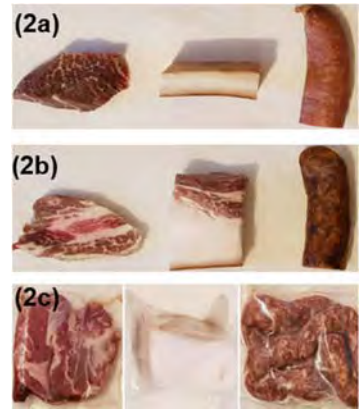

Fig. 2. Pork products tested for authenticity using DNA markers

The amplification of genomic regions from *SLC45A2* (exon 3 and 4) and *MC1R* (exon 1) genes was performed from DNA samples purified from reference blood samples or pork products with the SsoAdvanced Universal SYBR Green Supermix (BioRad, USA). Three primer sets specific for these genomic regions were designed from the NCBI pig reference genome (GCF_000003025.6).

The PCR was performed in 20 µl reactions, containing 10 pmol of specific primers and 50 ng of genomic DNA. The amplification was performed at: 94 °C for 2 min 1 cycle; 35 cycles of 94°C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec. To assess the quality of the PCR amplicons, 5 µl of each reaction volume was analysed on a 2% agarose gel, containing 1X SybrSafe (Invitrogen, USA) and in 1X TBE buffer (Lonza, Belgium). The sequencing reactions were performed with the same

PCR primers and using the BigDye™ Terminator v3.1 Cycle Sequencing Kit. After purification and denaturation with formamide, the sequencing products were analysed on a SeqStudio equipment (Thermo Fisher Sci, USA). The sequencing chromatograms were analysed using BioEdit (https://thalljiscience.github. io/).

RESULTS AND DISCUSSIONS

Gel electrophoresis analysis of the PCR amplification reactions, targeting some genomic regions from *SLC45A2* (exons 3 and 4) and *MC1R* (exon 1) genes from pig and wild boar reference samples and pork products, evidenced the presence of specific amplicons (Figure 3).

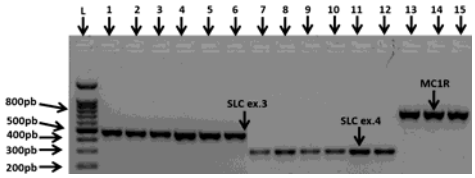

Fig. 3. Agarose gel electrophoresis profile highlighting amplification products obtained from *SLC45A2* and *MC1R* genes using pigs and wild boar reference samples and some pork products. L- 100 bp ladder GeneDireX

The comparative analysis of the sequencing chromatograms obtained from exons 3 and 4 of the *SLC45A2* gene from red and blond Mangalitza, Large White and Romanian wild boar, allowed the evaluation of polymorphisms at the codons 269 and 319 (Figure 4), involved in pigmentation [9].

All red Mangalitza individuals analysed were carriers of a haplotype called SLC-1, which is characterized by the presence of two SNP in the codons 269 (c.806G) and 319 (c.956A). These SNPs cause two amino acid substitutions in the protein encoded by the *SLC45A2* gene p.Gly269 and p. His319 (Figure 4).

All blonde Mangalitza individuals analysed were carriers of a haplotype called SLC-2. It is characterized by the presence of two SNP in the codons 269 (c.806A) and 319 (c.956G), which are associated with two amino acid substitutions in the protein encoded by the *SLC45A2* gene p.Glu269 and p.Arg319 (Figure 4).

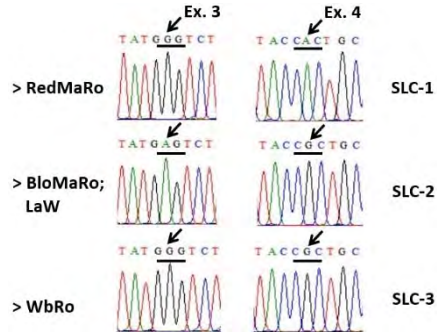

Fig. 4. Part of the sequencing chromatograms obtained from the *SLC45A2* gene (exons 3 and 4) highlighting haplotypes identified in red and blonde Mangalitza, Large White and Romanian wild boar

The results are consistent with the previous studies, according to which *SLC45A2* gene mutations are associated with the red and blonde phenotype in Mangalitza breed [9].

Surprisingly, in Large White pigs the SLC-2 haplotype was identified with high frequency, although the phenotype is white not blonde as in Mangalitza (Figure 4). These phenotypic differences in colour could be explained by the fact that in Large White pig, mutations in the *KIT* and *MC1R* genes, which are associated with white colour [7, 10, 11], most likely have a dominant effect on *SLC45A2* gene.

All Romanian wild boar analysed were carriers of a haplotype called SLC-3, which was absent in individuals within the analysed pig breeds. It is characterized by the presence of two SNP polymorphisms in codons 269 (c.806G) and 319 (c.956G), which are associated with two amino acid substitutions in the protein encoded by the *SLC45A2* gene *i.e.* p.Gly269 and p. Arg319 (Figure 4).

The comparative analysis of the sequencing chromatograms obtained from

the *MC1R* gene (exon 1), evidenced that all red and blonde Mangalitza and Romanian wild boar individuals analysed are carriers of the wild type E^+ allele. This is characterized by the presence of a guanine in the codon 124 (p.Asp124). Large White individuals were carriers a non-E^+ allele (E^P) (Figure 5), which is consistent with literature data [7].

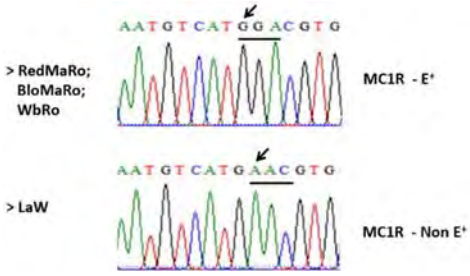

Fig. 5. Part of the sequencing chromatograms obtained from *MC1R* gene (exon 1) highlighting a mutation that differentiate Mangalitza and Romanian wild boar from Large White pigs

The comparative analysis of the results obtained by sequencing of some exonic regions from *SLC45A2* and *MC1R* genes in reference pig and wild boar samples, evidenced that using a combined information from these SNPs (Figure 4 and Figure 5), it is possible to differentiate red and blonde Mangalitza or Mangalitza from Large White and Romanian wild boar.

Using this approach, we analysed some pork products for these genetic markers, in order test the possibility to differentiate Mangalitza from commercial pigs (e.g. Large White) or wild boar. The results are shown in the chromatograms obtained by sequencing of *SLC45A2* (exons 3 and 4) and *MC1R* (exon 1) genes (Figure 6).

The products from producer P1 have been declared to be obtained from Large White pork. Genetic analyses have shown that they carry the SLC-2 haplotype for the first marker (Figure 6).

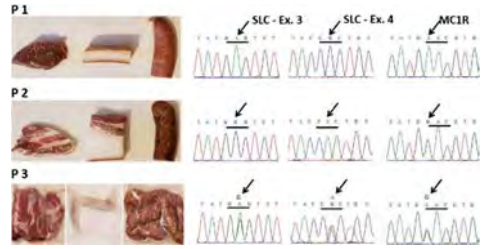

Fig. 6. Part of the sequencing chromatograms obtained from *SLC45A2* and *MC1R* genes highlighting SNP polymorphisms, which makes possible the differentiation of Mangalitza from commercial pigs in pork products

The comparison of these results with those obtained in the reference samples (Figure 4) evidenced that the products could come from Large White pigs, but also from the blond Mangalitza, as they are carriers of the same SLC-2 haplotype. However, the analysis of *MC1R* marker in these products revealed the presence of a non-E^+ allele (E^P), found in some commercial pig breeds (e.g. Large White) (Figure 6). The results are consistent with the label provided by the manufacturer.

The products from producer P2 have been declared to be obtained from red Mangalitza pork. Genetic analyses have shown that they carry the SLC-1 haplotype for the first marker (Figure 6). According to the reference samples (Figure 4), these products could have been produced from red Mangalitza pork. Analysis of the *MC1R* marker in these products revealed the presence of E^+ allele (Figure 6), found in Mangalitza breed or wild boar (Figure 5). However, the presence of wild boar in these products is excluded due to the presence of SLC-1 haplotype found in Mangalitza. In Romanian wild boar SLC-3 haplotype is present (Figure 4). The results are consistent with the label information provided by the manufacturer.

The products from producer P3 have been declared to be obtained from red Mangalitza pork. Genetic analyses have shown that they are heterozygous SLC-1 /

SLC2 for the first marker (Figure 6). The SLC-1 haplotype indicates the presence of meat from red Mangalitza and the SLC-2 haplotype indicates the presence of blond Mangalitza or from other pig breed (e.g. Large White). Analysis of the *MC1R* marker in these products revealed the presence of both alleles in heterozygous condition: a non-E^+ allele (E^P), found in some commercial pig breeds (e.g. Large White) and a E^+ wild type allele, which is present in Mangalitza breed and wild boar (Figure 6). However, the use of Romanian wild boar meat in these products is excluded due to the absence of SLC-3 haplotype. The results evidenced that the analysed products (meat and bacon) were probably obtained from hybrids between Mangalitza and other commercial pig breed or in the case of the minced meat product (sausage) from mixtures of meat.

CONCLUSIONS

The DNA analysis revealed that the SNPs identified in the *SLC45A2* and *MC1R* genes could be used as genetic markers to differentiate red and blonde Mangalitza or from Romanian wild boar and commercial pigs breed (e.g. Large White) in biological samples and in some meat products.

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