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**Simon, Á., Tisza, Á., Csikós Á, Jávor, A., Czeglédi, L.**

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**Poljoprivredni fakultet u Osijeku, Poljoprivredni institut Osijek**

Faculty of Agriculture in Osijek, Agricultural Institute Osijek

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# DETECTION OF PIG MEAT, LIVER AND LARD IN BEEF BY CE-SSCP

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Original scientific paper

***SUMMARY***

***Identification of animal species from foodstuffs is important in order to identify frauds to prevent substitutions and admixtures in animal products. In this paper we demonstrate the identification of cattle and pig species by polymerase chain reaction (PCR) capillary electrophoresis - single stranded conformation polymorphism (CE-SSCP) method. The procedure is based on the amplification of the 12S rRNA gene encoded in the mitochondrial DNA (mtDNA). Since mtDNA copy number is highly tissue dependent mixtures of different pig tissues in cattle meat were prepared, at a concentration of 1, 5, 10, 20 w/w% of pig lard, liver and loin. It was determined that regardless the tissue type pig DNA can be detected by CE-SSCP at each contamination level.***

***Key-words: CE-SSCP, mtDNA, species identification, 12S rRNA***

## INTRODUCTION

Species identification in beef products has always been important for both the consumers and producers, because of economical, health and religious issues. Adulteration of beef with products from cheaper counterparts is a constant problem of the food industry nowadays. Despite the European Union strict labelling system can be easily evaded with mislabelling. There is a constant need for genetic traceability of food products for fraud detection. Traditional species identification methods are protein-based, including isoelectric focusing (IEF) and immunological methods. IEF separate proteins by their isoelectric point and result in a species specific protein pattern. Drawback of the method is that the obtained results are influenced by temperature and duration of the heat treatment during the technological process. IEF patterns can be too complex and interpretation of the results is difficult when multiple species are present in the sample (Skarpeid et al., 1998). Antibodies, mainly monoclonals can also be used to detect species. Chen et al. (1998) successfully produced monoclonal antibodies against pig thermal-stable muscle proteins with a detection limit of 10% pork in raw and cooked meat as well. However, finding protein antigen for species identification is challenging because fewer species-specific protein marker exist compared to DNA based markers. Production of monoclonal antibodies is also labour intensive and expensive process while the use of polyclonals can be affected by cross-reaction with closely related proteins. On the contrary, DNA-based methods can be characterized with specificity, sensitivity and high reproducibility. DNA is a macromolecule not affected by heat or chemical degradation and less affected by mechanical stress during food processing compared to proteins (Dalvit et al. 2007). DNA can be selectively amplified with PCR while protein amplification method does not exist. Methodologies that target mitochondrial DNA have the following advantages over methodologies targeting genomic DNA: mtDNA is present in much larger copy number compared to gDNA, it improves the possibility to be amplified during PCR and mtDNA has higher mutation rate which induces substantial genetic interspecies variation. The number of mitochondria may vary dramatically in different cell types and physiological conditions. Copy number estimations of the mtDNA in different cells mainly derived from human studies. A normal liver cell contains ~8000, a myocardium ~7000, a skeletal muscle cell ~3700, an adipocyte ~300 while a pancreatic cell contains ~100 copy of mtDNA respectively (Miller et al. 2003;

*M.Sc. Ádám Simon (simon.a@agr.unideb.hu), M.Sc. Ákos Tisza, M.Sc. Ádám Csikós, Prof. Dr. András Jávor, Assist. Prof. Levente Czeglédi - University of Debrecen, Faculty of Agriculture, Food Science and Environmental Management, Department of Animal Science, 138. Boszormenyi Street, H4032 Debrecen, Hungary*

Yin et al. 2004; Kaaman et al. 2007). Bellagamba et al. (2001) applied restriction site analysis of PCR products of cytochrome b to discriminate species in meat meal and animal feedstuffs. They used *Bst*N I digestion for species-specific fragment for cattle identification against pig. The principle of the PCR-SSCP technique is that single stranded DNA molecules take on sequence dependent three-dimensional structure after denaturation. Single-stranded molecules under non-denaturing condition differing by as little as a single base substitution can form different conformers and migrate differently in a non-denaturing gel. In our PCR CE-SSCP method, a 12S rRNA mtDNA fragment is amplified using fluorescently labelled primers and separated via capillary electrophoresis. We chose CE-SSCP method due to its simplicity and higher analysis speed because there are no further enzymatic steps involved after PCR compared to RFLP. It can also be carried out on a standard vertical electrophoresis unit. Disadvantage of the method is that reference samples from known origin must be investigated alongside with the unknown sample.

## MATERIALS AND METHODS

Different pig tissues were prepared at concentrations of 1, 5, 10, 20 w/w% of pig lard, liver and loin for detection in cattle meat (Table 1). Samples 1-4 were used as controls. Total mass of the samples were 100±1 mg. Homogenization of the samples was performed with an Ultra Turrax T10 rotor-stator (IKA).

**Table 1. Food samples and food sample mixtures used in our tests**

| Sample | Component | Sample | Component |
|---|---|---|---|
| 1 | 100% pork bacon | 9 | 80% cattle spare ribs 20% pork liver |
| 2 | 100% pork liver | 10 | 90% cattle spare ribs 10% pork liver |
| 3 | 100% pork loin | 11 | 95% cattle spare ribs 5% pork liver |
| 4 | 100% cattle spare ribs | 12 | 99% cattle spare ribs 1% pork liver |
| 5 | 80% cattle spare ribs 20% pork bacon | 13 | 80% cattle spare ribs 20% pork loin |
| 6 | 90% cattle spare ribs 10% pork bacon | 14 | 90% cattle spare ribs 10% pork loin |
| 7 | 95% cattle spare ribs 5% pork bacon | 15 | 95% cattle spare ribs 5% pork loin |
| 8 | 99% cattle spare ribs 1% pork bacon | 16 | 99% cattle spare ribs 1% pork loin |

mtDNA was selectively isolated with a method described by Das et al. (2012). In the final step DNA was resuspended in 50$\mu$l double distilled water. Concentration and quality of the isolated mtDNA samples were determined using NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, USA). Nucleotide sequences of 12S rRNA mitochondrial gene of pig (AM158316.1) and cattle (GQ926965.1) were obtained from the NCBI GenBank database. Nucleotide sequences were then aligned using CLUSTAL OMEGA algorithm to check for conservative regions. After that primers forward primer 5′-ACTCTAAGGACTTGGCGGTG-3′ and reverse primer 5′-TTTACTGCTAAATCCTCCTT-3′ were picked with Primer3 software. Following primers targeting RYR1 gene (NC_010448) were designed with Primer3 software too: forward 5′-AGACCTTTCTCTTTGACCTTGAT-3′ and reverse 5′-CCAGACCTGGTGACATAGTTGA-3′. After that polymerase chain reaction (PCR) targeting RYR1 gene was performed to check for gDNA contamination which may interfere with the mtDNA concentration measurement. PCR of the 12S rRNA was performed in 10 $\mu$l volume containing 10x Dream Taq buffer (Fermentas, USA), 200 $\mu$M dNTP mixture (Fermentas, USA), 4 mM $MgCl_2$ (Promega, USA), 2 pmoles of FAM labelled forward primer (Sigma, Germany), 2 pmoles of VIC labelled reverse primer (Sigma, Germany), 1U Dream Taq polymerase (Fermentas, USA) and 150 ng DNA template. PCR was carried out in a PTC-200 thermal cycler (Bio-Rad). Thermal profile was: 95°C for 1.5 min followed by 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The final extension step was 5 min at 72°C. Amplified PCR products were analysed in 1.5 m/v% agarose gel (Lonza, France) for 1h at 6V/cm in TAE (Lonza, France) buffer and stained with ethidium-bromide (Applied Biosystems, USA). The samples were prepared for capillary electrophoresis analysis as follows: total volume of 10 $\mu$l consisted of 0.5 $\mu$l, 2 fold diluted PCR product, 0.5 $\mu$l LIZ 500 size standard and 9 $\mu$l of HiDi formamide (Life Technologies, USA). Capillary electrophoresis was performed on ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA), equipped with an argon-ion laser, emitting light at 488-514 nm. Samples were electrokinetically -injected at 15 kV for 4 sec to a 47 cm (effective length: 30 cm) long 50 $\mu$m diameter (Applied Biosystems) capillary filled with 15 w/t% solution of Pluronic F108 polymer (Sigma, Germany) according to Hwang et al. (2013) containing 0.7x Genetic Analyzer buffer (Applied Biosystems, USA). Electrophoresis was performed at 35°C with 15 kV and 40 min running time. Signal detection was between 525-650 nm. Raw data were collected using the Data Collection software 3.1.0., and processed with GeneMapper® 3.7 (Applied Biosystems) software (Figure 2). To obtain reproducible results, electropherograms were calibrated by fixing the positions of peaks produced by the LIZ 500 size standard (Applied Biosystems, USA).

## RESULTS AND DISCUSSION

Figure 1 shows that 12S rRNA PCR products were detected while gDNA RYR1 PCR products were not detected. The absence of gDNA was shown (Figure 1) and subsequently, it could be rough parameter predictor for estimation of mtDNA copy number in a different pig tissue types (Table 2).

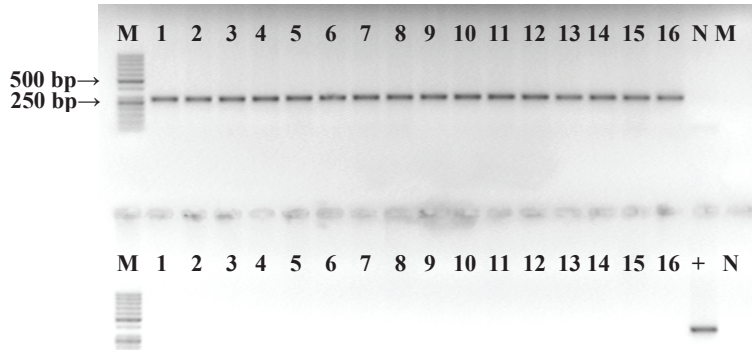


**Figure 1. Agarose gel electrophoresis of the labelled PCR products. Upper part shows a single PCR products (283 bp) with labelled primers, lower part shows the PCR products (329 bp) of RYR1 primers. gDNA contamination was not detected. Lanes represents samples 1-16 as defined in the material and methods. +: positive control N: negative control; M: 50 bp ladder (Thermo Fischer Scientific, USA).**

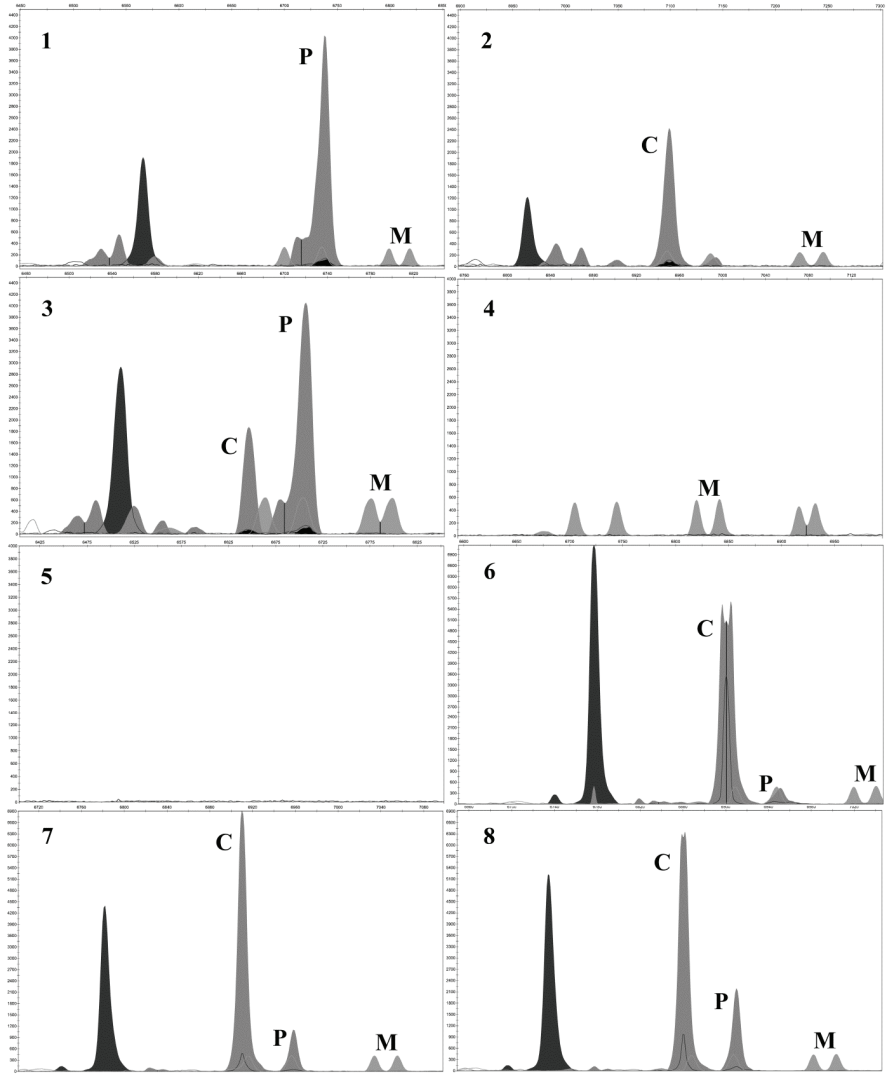


**Figure 2. CE-SSCP electropherograms representing species-specific patterns of 12S rRNA of the pig (P) and cattle (C) samples. M: LIZ 500 DNA marker. VIC and 6-FAM labelled strands are shown as light grey and black peaks. Conformation changes were only detectable in the VIC labelled conformers. Vertical axis represents relative fluorescents units (RFU); the horizontal represents data points (1 data point is equal to 220 msec migration time). 1: pig control; 2: cattle control; 3: cattle + pig control; 4: LIZ standard control; 5: negative control; 6: 99% cattle loin + 1% pig lard; 7: 99% cattle loin + 1% pig loin; 8: 99% cattle loin + 1% pig liver**

**Table 2. Estimation of mtDNA copy number in different tissues, based on our spectrophotometric data**

| Tissue type | NanoDrop concentration measurement (ng/$\mu$l) | Calculated amount (ng) of mtDNA in 1 mg tissue | Rough estimation of mtDNA copy number in 1 mg tissue |
|---|---|---|---|
| Pig liver | 2473.58 | 1236.79 | 6.87*10^10 |
| Pig loin | 506.57 | 1.41 | 1.41*10^10 |
| Pig lard | 117.91 | 58.9 | 3.27*10^9 |

Calculations in Table 2 are based on the assumption that the average weight of a base pair is 650 g/mol and 16679 base pair is the length of the pig mtDNA (Ursing et al., 1998). According to this estimation, even 1 mg tissue contains abundant amount of template for PCR. Figure 2 shows clear separation of the pig and cattle specific bands in the control runs as well as in the test runs. We determined that this method can detect as low as 1 w/w% pig lard (Figure 2 /6/), loin (Figure 2 /7/) and liver (Figure 2 /8/) mixed with cattle loin. It should be mentioned that our method is not suitable for quantitative estimations of the species in the starting material due the fact that the mtDNA copy number is highly depended on tissue types, which can lead to dissimilar peak intensities (Figure 2).

## CONCLUSION

In summary, a 12S rRNA based PCR CE-SSCP method was developed to identify pig and cattle species in the test samples. 1% pig mtDNA was detectable in all cases (cattle meat mixed with pig lard, pig loin and pig liver). Further aim of the experiment is to investigate the applicability of the method with the described primers by involving additional mammalian species and commercially available processed beef products to the analysis.

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## REFERENCES

1. Bellagamba, F., Moretti, V.M., Comincini, S. Valfrè, F. (2001): Identification of species in animal feedstuffs by polymerase chain reaction-restriction fragment length polymorphism analysis of mitochondrial DNA. Journal of Agricultural and Food Chemistry, 49(8): 3775-3781. doi: http://dx.doi.org/10.1021/jf0010329
2. Chen, F., Hsieh, Y.P., Bridgman, R.C. (1998): Monoclonal Antibodies to Porcine Thermal-Stable Muscle Protein for Detection of Pork in Raw and Cooked Meats. Journal of Food Science, 63(2): 201-205. doi: http://dx.doi.org/10.1111/j.1365-2621.1998.tb15709.x
3. Dalvit, C., De Marchi, M. Cassandro, M. (2007): Genetic traceability of livestock products: A review. Meat Science, 77(4): 437-449. doi: http://dx.doi.org/10.1016/j.meatsci.2007.05.027
4. Das, S.K., Sengupta, D., Ghosh, D., Dutta, D. (2012): An innovative method of cellulose acetate membrane based isolation of mitochondria and mtDNA extraction from the liver of *Duttaphrynus melanostictus* (Schneider, 1799). Indian Journal of Biotechnology, 11(2): 171-175. Retrieved from: http://nopr.niscair.res.in/handle/123456789/14015
5. Hwang, H.S., Shin, G.W., Park, H.J., Ryu, C.Y. Jung, G.Y. (2013): Micellar ordered structure effects on high-resolution CE-SSCP using Pluronic triblock copolymer blends. Electrophoresis, 34(4): 518-523. doi: http://dx.doi.org/10.1002/elps.201200520
6. Kaaman, M., Sparks, L., Van Harmelen, V., Smith, S., Sjölin, E., Dahlman, I. Arner, P. (2007): Strong association between mitochondrial DNA copy number and lipogenesis in human white adipose tissue. Diabetologia, 50(12): 2526-2533. doi: http://dx.doi.org/10.1007/s00125-007-0818-6
7. Miller, F.J., Rosenfeldt, F.L., Zhang, C., Linnane, A.W., Nagley, P. (2003): Precise determination of mitochondrial DNA copy number in human skeletal and cardiac muscle by a PCR-based assay: lack of change of copy number with age. Nucleic Acids Research, 31(11): e61. doi: http://dx.doi.org/10.1093/nar/gng060
8. Skarpeid, H., Kvaal, K. Hildrum, K.I. (1998): Identification of animal species in ground meat mixtures by multivariate analysis of isoelectric focusing protein profiles. Electrophoresis, 19(18): 3103-3109. doi: http://dx.doi.org/10.1002/elps.1150191811
9. Ursing, B.M., Arnason, U. (1998): The complete mitochondrial DNA sequence of the pig (*Sus scrofa*). Journal of Molecular Evolution, 47(3): 302-306. doi: http://dx.doi.org/10.1007/PL00006388
10. Yin, P., Lee, H., Chau, G., Wu, Y., Li, S., Lui, W., Wei, Y., Liu, T., Chi, C. (2004): Alteration of the copy number and deletion of mitochondrial DNA in human hepatocellular carcinoma. British Journal of Cancer, 90(12): 2390-2396. doi: http://dx.doi.org/10.3748/wjg.v19.i47.8880

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