# Animal welfare, etológia és tartástechnológia


# Animal welfare, ethology and housing systems

Volume 11 Issue 2

Gödöllő
2015

# SPECIES IDENTIFICATION IN MEAT AND CHEESE PRODUCTS BY PCR-SINGLE STRAND CONFORMATION POLYMORPHISM (PCR-SSCP) AND DNA SEQUENCING

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**Abstract**

In recent years species identification in foodstuffs by molecular biological methods have received more attention than in the earlier decades. The food adulteration scandals have highlighted to the importance of species identification techniques.
In our study foodstuffs were analysed by PCR-SSCP and DNA sequencing techniques. Samples were originated from hypermarkets. DNA was extracted from foodstuffs. After DNA isolation we have amplified 278 bp region of 12S rRNA gene of mitochondrial genome. Amplified fragment was denaturated by high temperature and presence of formamide before SSCP. Denaturated PCR amplicons were separated by non-denaturing polyacrylamide gelelectrophoresis. DNA bands were visualized by silver-staining method. After PCR-SSCP analysis the non-denaturated PCR amplicons of 12 meat and 6 cheese products were analyzed by DNA sequencing.
After PCR-SSCP analysis 6 meat products of 12 samples and 3 cheese products of 6 samples revealed to contain undeclared species. These results were confirmed by DNA sequencing that it showed the same results in the same samples. These results demonstrated that PCR-SSCP method is a reliable technique for species identification analysis in foodstuffs. Furthermore the PCR-SSCP method is a low cost method compared to than DNA sequencing or real-time PCR techniques.
**Keywords**: species identification, PCR-SSCP, DNA sequencing

**Fajazonosítás hús- és sajttermékekből PCR-egyszálú DNS konformáció polimorfizmus (PCR-SSCP) és DNS szekvenálás alkalmazásával**

**Összefoglalás**

Az elmúlt években az élelmiszerekből molekuláris biológiai módszerekkel történő fajazonosítás kiemelkedő fontosságúvá vált. Az Európai Unió területén bekövetkező élelemiszerhamisítási botrányok világítottak rá ezeknek a módszereknek a fontosságára.
PCR-SSCP és DNS szekvenálás alkalmazásával vizsgáltunk kereskedelmi forgalomból származó élelmiszereket. Ezekből a termékekből izoláltunk DNS-t, majd a mitokondriális 12S rRNS gén 278 bp hosszúságú szakaszát szaporítottuk fel. Az amplikonokat magas hőmérsékleten és formamid jelenlétében egyszálúsítottuk, majd nem denaturáló közegben, eltérő konformációjuk alapján poliakrilamid gélen választottuk el. A gélen lévő DNS sávokat ezüstfestéssel tettük láthatóvá. A vizsgált 12 hús- és 6 sajttermék esetében a PCR-SSCP módszer elvégzése után DNS szekvenálást is végeztettünk.

10.17205/SZIE.AWETH.2015.2.78

A PCR-SSCP vizsgálat alapján megállapítottuk, hogy 12 hústermékből 6, 6 sajttermékből 3 tartalmazott a címkézésen nem jelölt fajt. Ezeket az eredményeket a DNS szekvenálás is megerősítette. Az eredmények a PCR-SSCP módszer alkalmazhatóságát bizonyítják. Továbbá a PCR-SSCP módszer alacsonyabb költségekkel jár, mint a DNS szekvenálás vagy a real-time PCR technika alkalmazása, így egy könnyebb és olcsóbb alternatívát teremt az élelmiszerekből végzett fajazonosítás végrehajtására.
**Kulcsszavak**: fajazonosítás, PCR-SSCP, DNS szekvenálás

## Introduction

Due to food adulteration scandals in member states of European Union species identification have received attention in last years. Because of these cases a number of researchers have developed molecular biology methods to identify species in foodstuffs. The protein-based methods and fatty-acid composition analyses have outstanding importance among molecular biology techniques. Immunology (*Richter et al., 1997; Hurley et al., 2004*); chromatography (*Mayer et al., 1997; Chou et al., 2007*) and electrophoretic methods (*Galvani et al., 2001; Rabilloud, 2002*) are widespread techniques, nevertheless the cost of recently developed DNA-based methods lower than protein-based methods and fatty-acid analysis (*Di Pinto et al., 2005*). Amongst the DNA-based methods polymerase chain reaction (PCR) and other attached methods such as restriction fragment length polymorphism (RFLP) (*Kocher et al., 1989*), single-strand conformation polymorphism (SSCP) (*Hayashi, 1999*), denaturing gradient gel electrophoresis (DGGE) (*Peters et al., 2010*) are useful techniques to identify species specific DNA markers. The DNA sequencing is the most accurate genotyping method but its cost much higher than above mentioned methods. Consequently PCR-based techniques are commonly used in species identification practice.

## Material and methods

In our study commercial meat and cheese products were analysed by PCR-SSCP and DNA sequencing. Food samples were obtained from Hungarian hypermarkets. Samples stored in cryotubes at -20°C until further analysis. DNA was isolated from 50 mg cheese and meat by phenol-chloroform method of *De et al.* (2011). After extraction, concentration and quality of DNA were measured by NanoDrop 1000 (Thermo Fisher Scientific, USA) spectrophotometer. Universal primers were designed to 12S rRNA mitochondrial gene, those amplify a 278 bp fragment. Nucleotide sequence data of 12S rRNA gene of cattle (GQ926965.1), goat (GQ926969.1), sheep (JQ622016.1) and buffalo (GU119953.1) were received from NCBI GenBank database. Sequences of four species were aligned by CLUSTAL OMEGA algorithm of European Bioinformatics Institute (EBI). Universal primers were designed as described in *Csikos et al.* (IN PRESS). The designed universal primers were tested by OligoAnalyzer software for self-dimer, hetero-dimer and hairpin structure. Amplification of DNA from cheese samples were prepared in 20 µl volume containing 4 mM MgCl2 (Fermentas), 200 µM dNTP mix (Fermentas), 10x Dream Taq buffer (Thermo Fischer Scientific), 2 pmoles reverse primer R (5' – TTTACTGCTAAATCCTCCTT – 3') (Sigma), 2 pmoles forward primer F (5' – ACTCTAAGGACTTGGCGGTG – 3') (Sigma), 1U Dream Taq polymerase (Thermo Fischer Scientific) and 50 ng DNA template. PCR amplifications were carried out in a Peltier Thermal Cycler PTC-200 (Bio-Rad) with the following conditions: initial denaturation at 95 °C for 1.5

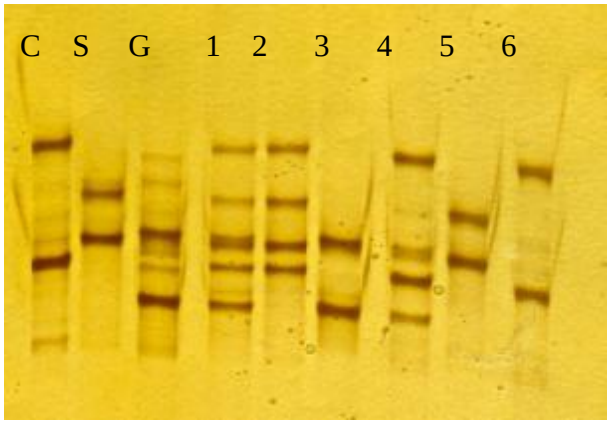
min; 35 cycles at 95 °C for 30s, 60 °C for 30s, 72 °C for 30s; final extension step at 72 °C for 5 min. Amplified PCR products were separated on 2 m/v% agarose gel (Lonza) for 1h at 100V in TAE (Tris-acetate-EDTA, pH: 8) (Lonza) buffer and stained by ethidium bromide solution (Thermo Fischer Scientific, USA). Before SSCP analysis, amplicons were heat-denatured in the presence of formamide. Single-strand PCR fragments were separated by non-denaturing polyacrylamide gel electrophoresis according to conformation differences. DNA bands were visualized by silver staining method (Merill et al., 1984) and were detected by UviproPlatinum gel documentation system. PCR products were purified by PCR Advanced™ PCR Clean Up System (Viogene, Taiwan) according to the manufacturer's instructions. Purified amplicons were sequenced by Macrogen Europe Inc. in Amsterdam, Netherlands. Nucleotide sequences were compared with reference sequences from NCBI GenBank database by alignment software (CLUSTAL OMEGA, EBI).

**Results and discussion**

According to our own recent study the extracted DNA suitable for PCR amplification and the designed universal primers are applicable for PCR-SSCP analysis to identify presence of polymorphisms amongst different livestock species for example cattle, goat, sheep and buffalo. After PCR-SSCP analysis we found that species specific single-strand conformers formed. Six commercial cheese samples were tested by PCR-SSCP and DNA sequencing methods. PCR-SSCP results shown 50% (3 of 6) of cheese samples contained undeclared species. In three cases labelled species were goat, two products declared as sheep milk cheese and one cheese sample contained cattle milk according to labelling. Two of three goat milk cheeses contained undeclared species, one of two sheep milk cheese contained undeclared cattle milk. The cattle milk cheese did not contain other species. Sheep, goat and cattle DNA extracted from milk were used as positive controls. The sheep pattern composed two nearby bands; the goat pattern made up of five bands, three light and two strong bands; the cattle pattern contained two strong, distant bands. On this basis, we found that species-specific patterns resulted by PCR-SSCP analysis. (*Figure 1.*). Meat and meat product samples were analysed by PCR-single strand conformation polymorphism and DNA sequencing methods. Following PCR-SSCP analysis 5 meat products of 12 samples revealed to contain undeclared species, such as chicken, duck and pig. Pig, chicken, turkey and duck pattern composed two strong distant bands. Goose pattern made up of four bands, two strong and two light bands. The patterns differed from each other. (*Figure 2.*). These results were confirmed by DNA sequencing that it showed the same results of meat products. These results demonstrated that PCR-SSCP method is a reliable technique for species identification analysis in foodstuffs. Furthermore the PCR-SSCP method is a low cost method compared to than DNA sequencing or real-time PCR techniques. This technique should be tested on other foodstuffs in further studies. DNA sequencing results verified these findings (*Table 1.*).

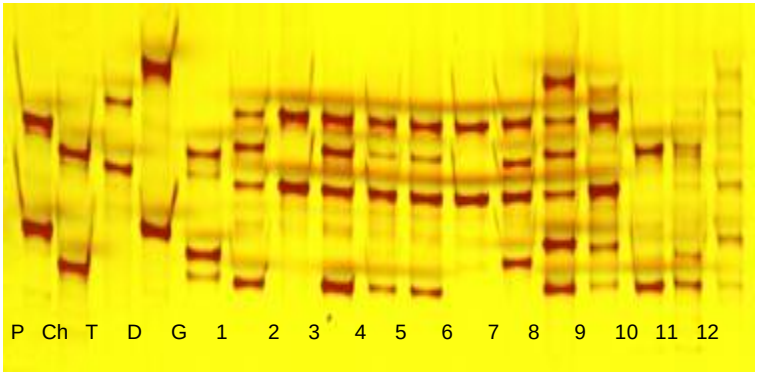
10.17205/SZIE.AWETH.2015.2.78

***Figure 1.*: PCR-SSCP pattern of 6 cheese samples**




C: cattle, S: sheep, G: goat, 1-6 cheese samples.

***Figure 2.*: PCR-SSCP pattern of 12 meat samples**




P: pig, Ch: chicken, T: turkey, D: duck, G: goose, 1-12.: meat samples.

The mitochondrial genome has a number of variable and conserved regions as well. This fact explains that selection of appropriate regions – 12S rRNA (*Dalmasso et al.*, 2004), cytochrome b (*Matsunaga et al.*, 1999), cytochrome oxidase subunit I. (*Kitpipit et al.*, 2014), D-loop region (*Lau et al.*, 1998) – can be used to design universal primers to authenticate foods of animal origin.

10.17205/SZIE.AWETH.2015.2.78

***Table 1*: Summarized results of PCR-SSCP and DNA sequencing analysis.**

| Number of products | Declared species | Detected species by PCR-SSCP | Detected species by DNA sequencing |
|---|---|---|---|
| 1. (cheese product) | **Goat** | **Goat, sheep, cattle** | **Goat, sheep, cattle** |
| 2. (cheese product) | **Sheep** | **Sheep, cattle** | **Sheep, cattle** |
| 3. (cheese product) | Goat | Goat | Goat |
| 4. (cheese product) | **Goat** | **Goat, cattle** | **Goat, cattle** |
| 5. (cheese product) | Sheep | Sheep | Sheep |
| **6. (cheese product)** | **Cattle** | **Cattle** | **Cattle** |
| 7. (meat product) | **Chicken** | **Chicken, Turkey** | **Chicken, Turkey** |
| 8. (meat product) | Turkey | Turkey | Turkey |
| 9. (meat product) | **Turkey** | **Chicken, Turkey** | **Chicken, Turkey** |
| 10. (meat product) | **Turkey** | **Chicken, Turkey** | **Chicken, Turkey** |
| 11. (meat product) | **Turkey** | **Chicken, Turkey** | **Chicken, Turkey** |
| 12. (meat product) | Turkey | Turkey | Turkey |
| 13. (meat product) | Turkey, Pig | Turkey, Pig | Turkey, Pig |
| 14. (meat product) | **Goose, Chicken, Poultry** | **Chicken, Turkey, Duck** | **Chicken, Turkey, Duck** |
| 15. (meat product) | Poultry | Chicken, Turkey, Duck | Chicken, Turkey, Duck |
| 16. (meat product) | Poultry | Chicken | Chicken |
| 17. (meat product) | Poultry, Pig | Pig, Turkey, Chicken | Pig, Turkey, Chicken |
| 18. (meat product) | Poultry, Duck | Duck, Chicken | Duck, Chicken |

## Conclusion

In this study six commercial cheeses were analysed by PCR-SSCP and DNA sequencing as well. The application of universal primers resulted a 278 bp amplicon of 12S rRNA mitochondrial gene of cattle, sheep and goat species. PCR-SSCP analysis of cow, goat, sheep and buffalo milk resulted species-specific patterns for each milk species. Analysis of commercial cheeses resulted the detection of food adulteration as cow DNA was detected in goat and sheep cheese and sheep and cow in goat cheese. DNA sequencing confirmed results of SSCP method. Based on our own results PCR-SSCP is a reliable method for milk products authentication. For further studies we suggest to develop and apply capillary electrophoresis SSCP to increase sensitivity of this method. (*García-Canas et al.*, 2004).

## Acknowledgement

This work was supported by the TÁMOP-4.1.1.C-12/1/KONV-2012-0014 project, Hungary.

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