Thesis of Ph.D. dissertation

SPECIES IDENTIFICATION FROM FOODSTUFFS BY THE DEVELOPMENT OF A PCR-SSCP METHOD

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1. Background and objectives of the doctoral thesis

Food scandals in recent years raised the attention of not only researchers having already been dealing with this issue, but that of the customers, as well, to a significant issue of food safety. Certain producers use cheaper ingredients during the production of their products to achieve higher profit. However, these producers mislabel these products indicating ingredients of higher costs, thus, the customer pays a higher amount of money for a product containing the given ingredient in a much lower concentration or in an extreme case, this product does not even contain this ingredient. It is a good example when pork is sold to the customer instead of beef. Food adulteration triggers further problems making the issue not a simple economic fraud, but health or in certain cases religious concerns, as well. As regards health issues, constituents not indicated in the label of a product may provoke heavy allergic reaction and concerning religious ones, it is known that people belonging to certain religious groups are not allowed to consume specific foodstuffs.

The issue above urged the researchers to develop various analytical methods. The development of these methods is continuous, aiming to create the most sensitive, widespread and cost-effective methods to screen out food adulteration. Analytical and molecular biological methods used to test foods of animal origin may be divided based on the type of material to be detected in three major categories: detection based on fatty acid composition and protein- and DNA-based techniques. From the three listed groups, the protein- and DNA based methods are the more widespread. Among the protein-based methods the specific immunologic, electrophoretic and chromatographic procedures worth highlighting, which enable detection of high accuracy, however, at a relatively high cost.

DNA-based methods represent the most recent field of food adulteration detection research (Plath et al., 1997). Most of the DNA-based methods depend on the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). The PCR reaction has a great advantage to be able to be combined with certain molecular genetic methodologies – currently, this is the single stranded DNA conformation polymorphism – making the investigation of the given gene of a given species to be more specific, thus, the effectiveness of species identification can be increased. In this case, we would like to investigate the possibilities of species identification from commercially available cheese, meat products and freshwater fish corpses. Besides, we would

like to test the possibilities of quantitative determination and in case of ripening cheese, we want to study the degree of degradation of DNA molecules.

During the research, I planned to perform the development of the PCR-single stranded DNA conformation polymorphism method to identify the species from foodstuffs. The aims of these experiments are listed below:

- the analysis of the sensitivity of the method for milk mixtures, as well as the testing of the applicability of PCR-SSCP method for commercially available cheese products,

- the analysis of the sensitivity of the method for meat mixtures, as well as the testing of the applicability of PCR-SSCP method for commercially available meat products,

- possibilities of the differentiation of Hungarian freshwater fish species by PCR SSCP and capillary electrophoresis method,

- the examination of the possibility of quantitative analysis for sheep and cattle species, as well as testing of the applicability of quantitative analysis for commercially available sheep-cattle cheese products.

2. Investigation of milk and milk products by PCR-SSCP method

To determine the sensitivity of the method, DNA mixtures with known concentration were applied, while to test the applicability of the PCR-SSCP method, commercially available cheese products were used. To amplify the DNA from the samples, universal primer pair was used.

In the planned experimental setup, DNA templates with identical concentrations were mixed in defined volumetric ratio. The settings for each series included two positive control samples, one of which contained 100 V/V % cattle DNA and the second sample included 100 V/V % sheep, goat and buffalo DNA, respectively. The mixture sequence consisted of samples containing 50, 40, 30, 20, 10 and 5 V/V % bovine DNA, and two positive controls. In case of the 5% v/v mixture ratio for each of the three series, the presence of bovine DNA was clearly demonstrated. Therefore, the percentage of the presence of bovine DNA was further reduced: the samples contained 5, 3, 1, 0.5 and 0.1 V/V % of bovine DNA relative to the DNA of the other species. The detection limit of this method was 3 V/V % in case of cattle-buffalo mixture and 5 V/V % for the cattle-sheep and cattle-goat mixtures. In case of the 3 V/V % ratio of cattle-buffalo mixture, the gel analyser software determined the intensity of the cattle-specific band to be 4.85% relative to the total band intensity. In this way, the presence of cattle can be objectively proven.

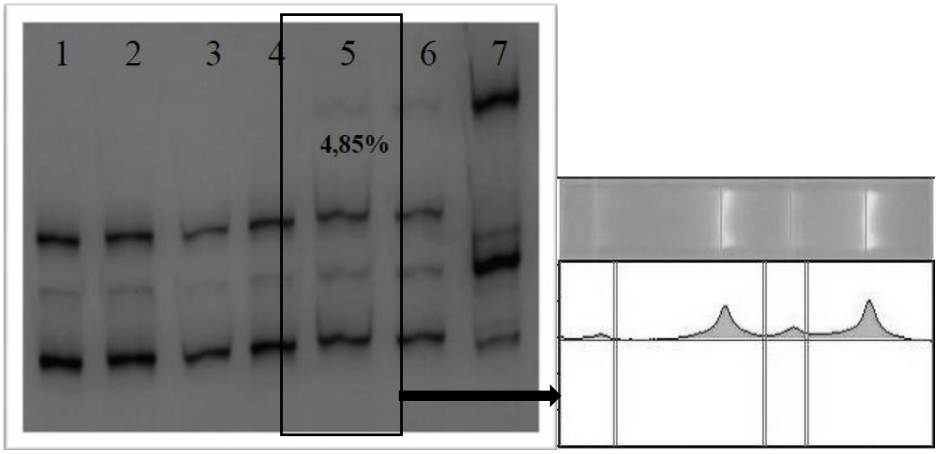

Figure 1: Result of polyacrylamide gel electrophoresis with cattle-buffalo DNA mixtures in the 0.1 V/V % - 5 V/V % range.

1: 100 V/V % buffalo DNA; 2: 0.1 V/V % cattle and 99.9 V/V % buffalo DNA, 3: 0.5 V/V % cattle and 99.5 V/V % buffalo DNA, 4: 1 V/V % cattle and 99 V/V % buffalo DNA, 5: 3 V/V % cattle and 97 V/V % buffalo DNA, 6: 5 V/V % cattle and 95 V/V % buffalo DNA, 7: 100 V/V % cattle DNA.

We tested the applicability of the method with commercially available dairy products. During the investigation, 39 Hungarian, 15 Swiss and 66 Bosnian cheeses were studied by PCR-SSCP method.

Table 1: Results of the PCR-SSCP analysis of 120 commercial cheese products

Declared species	Number of samples	Identified species by PCR-SSCP
Sheep	66	39 sheep
		15 sheep + cattle
		12 cattle
Cattle	18	18 cattle
Goat	22	15 goat
		7 goat + cattle
Sheep + cattle	13	11 sheep + cattle
		1 sheep
		1 cattle
Goat + cattle	1	1 goat + cattle

36 out of our 120 commercially available cheese products clearly showed the presence of non-labelled species, which is exactly a rate of 30%. 27 out of 66 sheep cheeses proved the presence of undeclared species, which is a rate of 40.9%. 7 out of 22 goat cheeses proved the presence of undeclared species, which is a rate of 31.82%. For 2 out of 13 sheep-cattle cheeses, the presence of undeclared species were detected, which is a rate of 15.38%. On the basis of the ones above, the highest difference was detected for the sheep cheeses. In 12 cases out of 66 sheep cheeses, only the presence of cattle were detected, however, this does not mean that these cheeses do not contain sheep DNA, but only that the presence of sheep DNA was unable to be detected at the given sensitivity. Somatic cell count of milk used during the production may affect the success of the detection of particular species.

3. Duplex-PCR reaction with sheep-cattle DNA mixtures – Investigation of the possibilities of quantitative detection

To set up a calibration equation to enable the estimation of the quantity of bovine DNA in the commercially available cheeses, sheep and bovine DNA of identical concentrations were mixed in specified ratios. The mixture sequences contained bovine DNA from 0.1 V/V % to 40 V/V % range relative to sheep DNA. PCR reactions made with the mixing series were performed in five replicates to determine the calibration equation of the mixture sequence. In each case, the intensity of each band related to each other was determined, and the relative intensity value for each mixing ratio was determined by the following formula.

$$\frac{I_{cattle}}{I_{cattle} + I_{sheep}} = Relative\ intensity$$

The formula for the determination of the relative intensity values

The averages of the relative intensity values were calculated. Upon the creation of the calibration equation, the average relative intensity values and the corresponding mixing ratios were considered to give a linear trend line of the calibration equation. The calibration equation was calculated from the measured band intensity values by LabWorks 4.0 (UVP, USA) software. In case of LabWorks 4.0, the results of the program are obtained as ratios. The resulting calibration equation can be expressed as y = 0.0933 + 0.0083x with a coefficient of determination (R^2) of 0.9067 showing a strong statistical relationship between the two variables. After obtaining the calibration equations, 25 commercially available sheep-cattle cheeses proven by previous tests to contain sheep and bovine DNA were tested by duplex PCR reaction. After the evaluation of the results by the LabWorks 4.0, the percentage of bovine DNA in 25 cheeses was between 4.68% and 77.68%.

4. Single cell gel electrophoresis

The single cell gel electrophoresis method is for the measurement of the degree of degradation of DNA within the cells. In this case, the method was used for cheeses ripened for longer periods of 6, 8, 10 and 18 months. The control was fresh milk. Result pictures were obtained by fluorescence microscopy. The detection of slides occurred by Nikon Eclipse 500

fluorescent microscope and Peltier-cooled Olympus DP72 camera. The applied lens had a magnification of 20, i.e. the total system has a magnification of 200. The slides were stained by ethidium-bromide. The result pictures were analysed by ImageJ 1.47 OpenComet plugin (NIH, USA).

Upon the examination of a cell with the ImageJ 1:47 Open Comet plugin, it was found that on the basis of the software, the proportion of the head of the comet was 77.79, while that of the tail was 22.21%. It is seen that the value for the tail is much less than the value for the head.

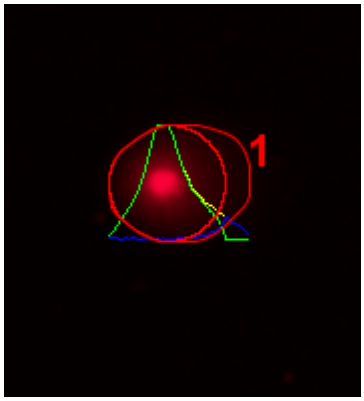

Figure 2: Picture of a compact tail for fresh milk sample detected by ImageJ 1.47 OpenComet plugin.

The rates were determined for a total of 85 cells by the OpenComet software. As a result it was found that the proportion of the head varied between 57.55 – 98.98%, while the proportion of the tail varied between 1.02 – 42.45%. The mean of the proportion of the head parts was 80.72%, with a standard deviation of 11.77%, while the mean of the proportion of the tail parts was 18.39% with the standard deviation of 11.77. In case of the long period ripened cheeses, no observable and assessable tail formation was found.

As during the single cell gel electrophoresis of the long period ripened cheeses there was no formation of tails, PCR reaction to be performed by universal primer pair was designed to detect the presence of DNA. In this case, universal primer pair designed for 12s rRNA gene and applied earlier was used.

Using the 12S rRNA universal primer pair, the specific DNA region was able to be amplified from all ripened cheese samples analysed during the single cell gel electrophoresis.

5. Investigation of meat and meat products by PCR-SSCP method

During the species identification from meat products, we studied mitochondrial 12S rRNA gene of 18 species – including human – with universal primer pair. The study included seven species of poultry, five species of mammals, five species of wild animals and human. Among poultry species domestic pigeon, chicken, guinea fowl, turkey, duck, Muscovy duck and goose were examined; among the domestic mammal species pig, cattle, buffalo, sheep, goat, horse and rabbit were included in the analysis; among wild animal species, the possibility of the differentiation of wild boar, mouflon, roe deer, red deer and fallow deer species were studied. Upon the differentiation of the involved species above by PCR-SSCP it was stated that, 16 out of the 18 species were able to be differentiated from each other on the basis of conformational differences in the single-stranded DNA. No detectable difference in the patterns i.e. in the conformation was found between sheep and mouflon as they are the same species. The same applied for the red deer and fallow deer. The different band patterns for the species are due to the differences within the same section. These differences trigger different conformations in the single strand DNA. The specific patterns obtained by PCR-SSCP analysis of the 18 species is shown in Figure 2.

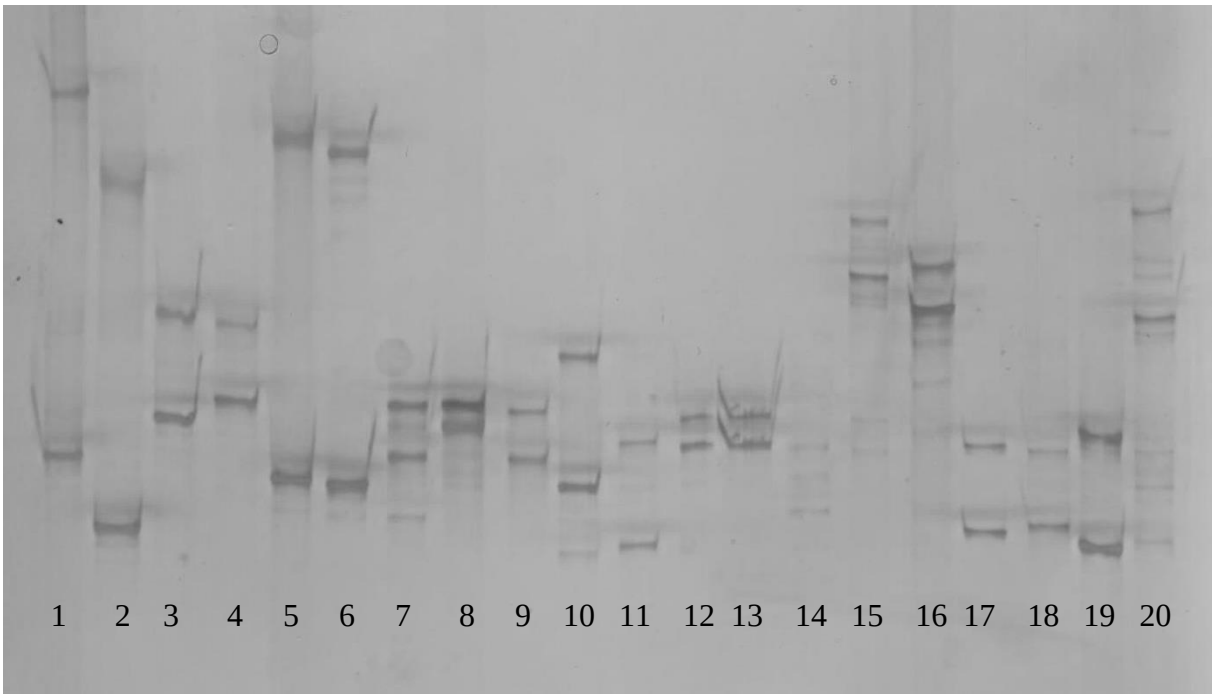

Figure 3: Identification of 18 species by PCR-SSCP.

Table 2: Common and scientific name of species examined by PCR-SSCP method with the same sequence as in the gel map shown above

Number of samples	Common name	Scientific name
1.	Domestic pigeon	*Columba livia domestica*
2.	Chicken	*Gallus gallus*
3.	Guinea fowl	*Numida meleagris*
4.	Turkey	*Meleagris gallopavo*
5.	Duck	*Anas platyrhynchos domestica*
6.	Muscovy duck	*Cairina moschata*
7.	Goose	*Anser anser*
8.	Pig	*Sus scrofa domestica*
9.	Wild boar	*Sus scrofa*
10.	Cattle	*Bos taurus*
11.	Buffalo	*Bubalus bubalis*
12.	Sheep	*Ovis aries*
13.	Mouflon	*Ovis aries orientalis*
14.	Goat	*Capra hircus*
15.	Horse	*Equus caballus*
16.	Domestic rabbit	*Oryctolagus cuniculus domestica*
17.	Red deer	*Cervus elaphus*
18.	Fallow deer	*Dama dama*
19.	Roe deer	*Capreolus capreolus*
20.	Human	*Homo sapiens*

The sensitivity of PCR-SSCP method was tested by poultry meat mixtures. Unified concentration of DNA of chicken and duck were mixed in defined ratios. The mixtures contained chicken DNA in 20 V/V %, 10 V/V %, 5 V/V %, 1 V/V % and 0.5 V/V % relative to duck DNA, respectively. The pattern of bands obtained by PCR-SSCP is shown in Figure 4.

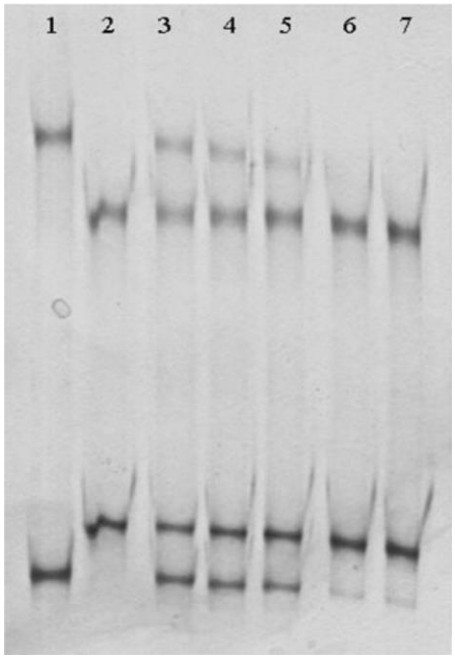

Figure 4: Results of polyacrylamide gel electrophoresis with chicken and duck DNA mixtures. 1: Positive control (chicken); 2: positive control (duck); 3: 20% chicken DNA; 4: 10% chicken DNA; 5: 5% chicken DNA; 6: 1% chicken DNA; 7: 0.5% chicken DNA.

In case of sample 7, there is a band indicating the presence of chicken, therefore, it can be stated that PCR-SSCP method has an appropriate sensitivity to detect the individual species, since in this case the presence of 0.5% was detected.

After testing the sensitivity of the method, its applicability for the species identification was investigated for 65 commercially available meat products. The products were made of the meat of poultry and mammalian species. Out of the 65 products, there were 20 turkey / chicken, 14 turkey, 11 pig, 8 chicken, 3 pig / cattle, 2 turkey / poultry, 1 turkey / chicken / poultry, 1 poultry, 1 duck / poultry, 1 horse / pig, 1 turkey / pork, 1 poultry / pork, 1 goose / pork products on the basis of the labels. PCR-SSCP analysis was performed for each product and the planned universal primers. The results are summarised in Table 3.

Table 3: Results of the PCR-SSCP analysis of 65 commercial meat products

Declared species	Number of samples	Identified species by PCR-SSCP
Turkey/chicken	20	20 turkey/chicken
Turkey	14	9 turkey
		5 turkey/chicken
Pig	11	11 pig
Chicken	8	7 chicken
		1 chicken/turkey
Pig/cattle	3	**2 pig**
		1 pig/cattle
Turkey/poultry	2	2 turkey/chicken
Turkey/chicken/poultry	1	1 turkey/chicken
Poultry	1	1 chicken
Duck/poultry	1	1 duck/chicken
Horse/pig	1	**1 horse/pig/cattle**
Turkey/pig	1	**1 turkey/chicken**
Poultry/pig	1	1 turkey/chicken/pig
Goose/pig	1	1 goose/pig

Altogether, out of the 65 commercial products examined, undeclared species were detected in 10 cases. This is 15.4% of the tested products.

Detection of porcine meat, liver and fat from beef by CE-SSCP

In this study, various pig tissues were used. Porcine fat, liver and muscle (pork chop) were mixed in 1, 5, 10 and 20 V/V % ratio with bovine meat (Table 4). Samples 1-4 were used as controls. The total mass of the samples was 100 ± 1 mg. The differentiation of each sample was performed by capillary electrophoresis system.

Table 4: Porcine and bovine mixtures

Sample	Ingredients	Sample	Ingredients
1	100% pork lard	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 lard	13	80% cattle spare ribs 20% pork chop
6	90% cattle spare ribs 10% pork lard	14	90% cattle spare ribs 10% pork chop
7	95% cattle spare ribs 5% pork lard	15	95% cattle spare ribs 5% pork chop
8	99% cattle spare ribs 1% pork lard	16	99% cattle spare ribs 1% pork chop

On the basis of the CE-SSCP results it was stated that this method is able to detect of the presence of porcine fat, liver and pork chop at a mixture ratio of 1 V/V % from bovine m. longissimus dorsi. However, this method is not suitable for the quantitative estimation of species from starting materials, since the copy number of mtDNA is strongly dependent on the type of tissues, resulting in different intensity peaks. Knowing all these it can be stated that the larger pieces of meat, the higher body weight, the more fat tissue and the thicker lard do not necessarily mean higher cell count, i.e. the copy number may be highly variable for a unit body weight.

6. Identification and differentiation of freshwater fish species by PCR-SSCP method

Upon the differentiation of the fish species from each other, two approaches were applied.. In the first approach, multiple primer pairs were designed for species groups where the goal was the differentiation within the groups, while in the other one, a universal primer pair was designed for the sequence of cytochrome oxidase subunit I (COI) genes for all of the investigated species.

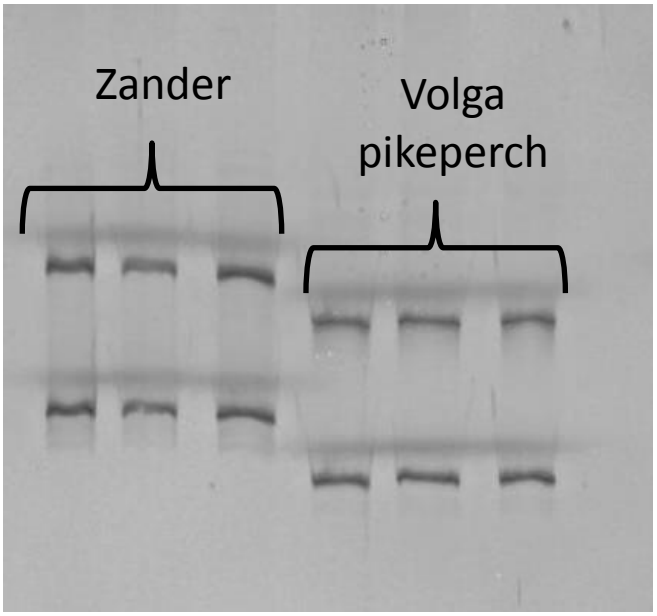

Figure 5: Differentiation of zander and Volga pikeperch by PCR-SSCP

Figure 5 shows the band patterns of zander and Volga pikeperch. It can be seen that there are well-distinguishable band patterns for both species, making possible their differentiation from each other.

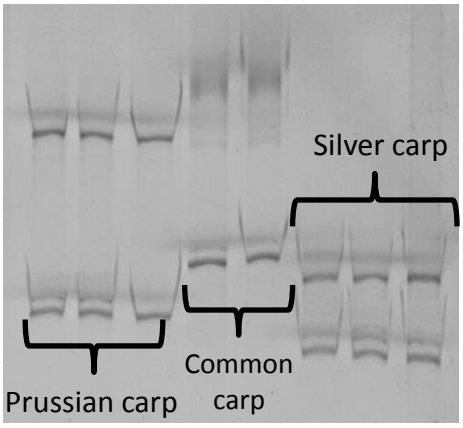

Figure 6: Differentiation of Prussian carp, common carp and silver carp by PCR SSCP

In Figure 6, the differentiation of Prussian carp, common carp and silver carp by PCR-SSCP is shown. It is seen that there are different band patterns for the three species, therefore, they can be distinguished from each other. In the pattern of the first two Prussian carp individuals, there is a pale band right above the bottom bands, which is unseen in case of the third individual. This phenomenon can be explained by the possibility that the single strand DNA molecules have more than one stable conformer.

In Figure 7, band patterns of 15 species can be differentiated. The samples 1, 2 and 3 were commercially available fish corpses and were labelled as rainbow trout, trout and freshwater trout on the product, respectively. Based on the results it can be stated that despite of the different name on the labelling, these samples are from the same species, as the same band patterns are observed. The rainbow trout is usually bred in fish breeding farms, therefore, it is of high possibility that all of the three samples represented this species. For the other species, unambiguously distinct band patterns are observed, i.e. these species are distinguishable from each other by PCR-SSCP system. The sample 8 shall be mentioned separately, as it showed as many as 3 bands. This is a good example to show that more stable conformers can be formed after the denaturation of the DNA.

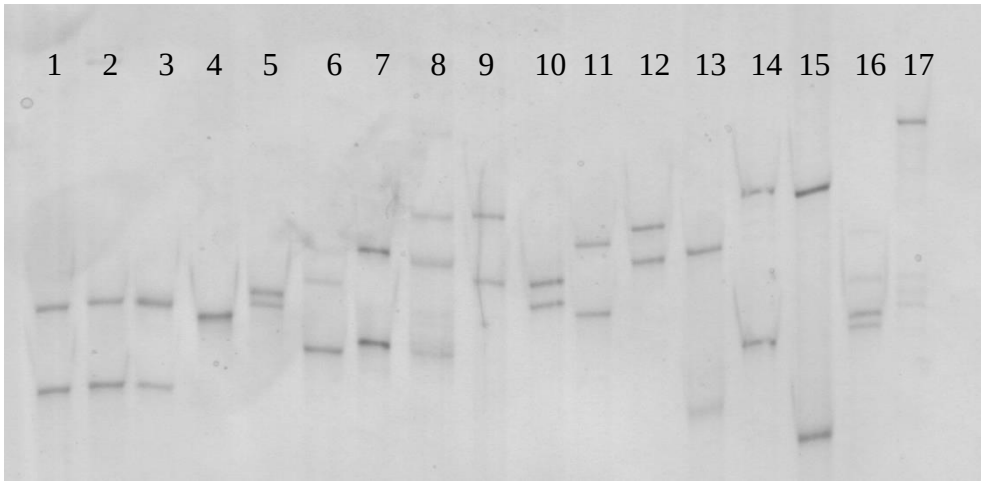

Figure 7: 1. rainbow trout, 2. trout, 3. freshwater trout, 4. catfish, 5. African catfish, 6. common carp, 7. Prussian carp, 8. silver bream, 9. common bream, 10. rudd, 11. bleak, 12. roach, 13. asp, 14. stone moroko, 15. tench, 16. zander, 17. northern pike

Altogether, 15 fish species were examined by PCR-SSCP system. Knowing the obtained results it can be stated that each of the 15 species was able to be differentiated from each other with the use of a single universal primer pair on the basis of conformational differences in denatured single-stranded DNA.

After performing the PCR-SSCP, universal primers with fluorescent labelling were also used for capillary electrophoresis – SSCP (CE-SSCP). Patterns of European catfish, African catfish, black catfish, goldfish, carp and rainbow trout species, which were obtained by CE-

SSCP method were compared. As a result of the analysis it was found that catfish and African catfish were unable to be differentiated, as they gave the same pattern in the SSCP-CE system, while the other species were able to be distinguished from each other on the basis of the band patterns.

Determination of carp DNA from digested corpses and maggots

The species-specific carp primers were tested for suitability in a way that carp meat was exposed to decay. As time progressed, maggots appeared on the meat sample, as well. The robustness of primers was demonstrated by that DNA was isolated from the rotten meat and from the maggots growing on the meat and afterwards, PCR reaction measurements were performed for the carp-specific primer pair using the extracted DNA as a template.

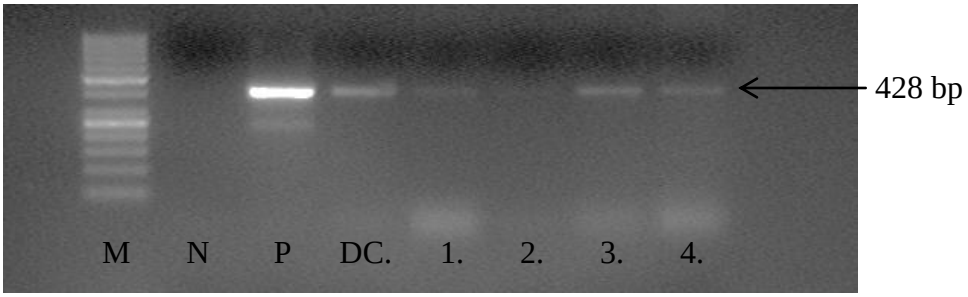

Figure 8: Results of PCR reaction performed with carp-specific primer pair and DNA from maggots. 1-4. Results of PCR reactions for DNA from maggots. M: 50 bp DNA ladder. N: negative control. P: positive control, DC: degraded carp.

The presence of carp DNA was successfully detected from DNA templates from maggots using a carp-specific primer, showing that the presence of a given species is able to be detected even from the feed of maggots adhered to their bodies or located at the beginning of their alimentary tract. Furthermore, the method proves the robustness of the carp-specific primer pair, as well, since it is able to propagate carp DNA also from a small quantity of sample.

7. Conclusions

The development of molecular genetics methods in the field of species identification from foodstuffs became of outstanding importance over the past two decades (Asensio et al., 2009).

Therefore, tests of molecular genetic methods were performed to develop methods for species identification from foodstuffs.

During our investigations, dairy products, meat products and fish species were differentiated by PCR single-stranded DNA conformation polymorphism. In the literature, there are few publications dealing with the examination of dairy products by the application of the PCR-SSCP method. Plath et al. (1997) tried to differentiate cattle, sheep, goat and buffalo species from milk by PCR-SSCP method, however, they did not find any difference in the band pattern for sheep and goat when goat and cattle DNA were mixed in 50-50 V/V % and 95-5 V/V %. As a result, they were able to detect both species in the case of 50-50% mixture, however, for 95-5% they did not succeed to do so. During the examination of dairy products, in our case a detection limit of 3-5% was achieved using the PCR-SSCP method. However, the use of other methods can decrease the detection limit. Rea et al. (2001) were able to distinguish cattle and buffalo species in mozzarella cheeses using duplex PCR with a detection limit of 1%. The examination of the same species was carried out in duplex PCR system by López-Calleja et al. (2005), as well. In their case, the detection limit was 0.1%, which is highly sensitive. It can be seen that the differentiation between two species with duplex-PCR is fairly simple and can be performed with high sensitivity, however, the disadvantage of the method compared with the PCR-SSCP is that only two species can be examined simultaneously and two primer pairs are necessary. The number of examined species can be increased by using multiplex PCR-test, however, with the increasing number of primer pairs, the design of primers becomes more complicated and the possibility to avoid cross-reactions also decreases. Bottero et al. (2003) performed the differentiation of sheep, goat and cattle species by multiplex-PCR. In their case, the detection limit was 0.5%. However, it should also be noted here, that the sensitivity of the method can be demonstrated, however, in practice such a small scale of adulteration does not have any economic benefit.

In case of our study on meat products, 18 species were able to be differentiated from each other by PCR-SSCP system. In their study Jürgen et al. (2001) performed the differentiation of 15 species. Similarly to our results, they also failed to differentiate pig from wild boar, which can be explained by the match of sequences, and they did not investigate mouflon, muscovy duck and guinea fowl. Sarri et al. (2014) studied the possibility of the differentiation of five species using the PCR-SSCP method. Four assemblies were used by them. In the first case, chicken and turkey, in the second, chicken, turkey and pork, in the third, cattle, pig,

chicken and turkey and in the fourth, cattle, pig, sheep, chicken and turkey were compared. As a result, it was found that all five species can be differentiated from each other. In their study, 12 meat products were analysed and in all cases, only the species declared in the labels were able to be detected. In contrast, in our study 10 out of the 65 meat products were detected to contain undeclared species. In our case, the detection limit was 0.5% (Tisza et al., 2016). Using duplex and multiplex-PCR reaction, a lower detection limit can be achieved similarly to the case of cheese products. Using a multiplex PCR reaction, Dalmasso et al. (2004) detected ruminant, poultry, fish and porcine meat from foods with a ratio of 0.002% related to the presence of pig. At higher, but still very low sensitivity, the presence of poultry and pig was able to be detected, reaching the detection limit of 0.01% (Krcmar and Rencova, 2003).

During the species identification by PCR-SSCP method, 15 freshwater fishes were successfully distinguished from each other. The target sequence was localised in the mitochondrial cytochrome oxidase subunit I, similarly to the differentiation of dairy and meat products where the target sequence was the sequence of the 12S rRNA gene of the mitochondrial genome. In cases of species identification of fishes, mitochondrial genes such as 12S rRNA (Asensio et al., 2001) and 16S rRNA (Khamnamtong et al., 2005) were the target. It should be highlighted regarding the studies of not only fishes, but of other animal groups and products that the PCR-SSCP method is highly sensitive to the differences in the DNA base pair and is able to detect a difference in a single base pair within a given section in the form of conformational changes (Oohara, 1997),. A further advantage of the method is that it is suitable for the study of degraded DNA, as well, since the required size of the PCR product should be not more than a few hundred base pairs (Rehbein et al., 1999).
Sriphairoj et al. (2010) studied nine species from the family Pangasiidae by PCR-SSCP method. During their work, five out of the nine species were able to be differentiated, however this group included as many as four economically important species. In another study, Schiefenhövel et al. (2013) performed the differentiation of seven species belonging to the family Sparidae by PCR-SSCP method. Five out of the seven species can be distinguished from each other, while in one case a difference within the species was observed. The determination of detection limit was performed in neither cases, only the differentiation was performed on the basis of the band pattern of the species, as occurred in our study.

Besides the qualitative determination - "there is - there is not" – the estimation or determination of the quantities of certain components is one of the most important challenges. We performed the quantitative detection of cattle based on the method described by Mafra et al. (2007) for cattle and sheep cheeses. We set up a calibration equation to make possible the estimation of the quantity of DNA. In the above mentioned study, the proportion of goat and cattle was determined in a similar way. In their study, the presence of cattle was between 9-13% in 17 cheeses, while this ratio was between 4-77% for 25 cheeses in our measurements. Although more precise quantification can be performed by real-time PCR method, its implementation is much more time-consuming, complex and entails higher material costs. Contrary to this fact, several studies can be found in the literature for real-time PCR quantification. Soares et al. (2010) investigated the qualification and quantification of pork in processed poultry products by a system using SYBR Green dye. The detection limit of pork DNA reached the limit of the previously described PCR duplex method, being 0.1% which is equal to 5 pg DNA.

To determine the degree of DNA degradation in foodstuffs, single cell gel electrophoresis was used. In this case, we used this method to test cheeses ripened for long periods, i.e. 6, 8, 10 and 18 months. The control was fresh milk. This method was originally developed for the investigation of mammalian cell cultures exposed to radioactive radiation (Ostling and Johanson, 1984). Subsequently, the effect of the radioactivity on foods was also investigated extensively (Verbeek et al., 2007; Khawar et al., 2010). For the investigation of food conserved by radiation, the European Union officially assigned the single cell gel electrophoresis (Internet reference 3.) as the officially accepted method. Cerda et al. (1998) studied the effect of time on chicken meat. Their study covered a shorter period than the one mentioned above for ripening cheeses. Fresh meat samples were held for 12 days at + 4°C and single cell gel electrophoresis measurement was carried out on days 1, 3, 7, 10 and 12. As time was passing by, the genomic DNA became more and more degraded. Initially, compact genome with no tail was found and later on there were long and diffuse tails. They detected even bacterial signals in the last test day. This result highlights the fact that heavily degraded genome can be found even after 12 days, while in the case of several months of aging only short DNA segments remain as it was shown by the result of the PCR reaction in the subsection Single cell gel electrophoresis of the section Results.

8. New scientific results of the thesis

Based on the results of my dissertation, the new scientific results of the thesis are listed below:

1. During the development of the PCR-SSCP study, the sensitivity of the method for milk and milk products was found to vary between 3-5 V/V %, related to bovine DNA. After the determination of the sensitivity of the method, its applicability was tested for altogether 120 commercially available samples. It was stated that in 36 out of the 120 products, the presence of undeclared species which were not mentioned by the manufacturer on the product label was clearly demonstrated, which represents 30% of the total samples.
To study the effect of ripening of cheeses on the genome of cells, single cell gel electrophoresis method was used. As a result, it was found that in case of fresh milk, compact tails were formed, while in case of cheeses ripened for 6, 8, 10 and 18 months, these structures are completely missing. In case of ripened cheeses, the PCR reaction performed with 12S rRNA universal primer pair resulted in a well-detectable PCR product.

2. When PCR-SSCP method was applied for meat products, the sensitivity of the method was also stated with the help of DNA isolates of chicken and duck with unified concentration and appropriate mixing ratio. 0.5% of chicken DNA was able to be detected when it was mixed with duck DNA. With the help of the method, 18 species out of the 20 were able to be distinguished from each other on the basis of the conformation differences in the single strand DNA. The applicability of the method was tested for meat products, as well. Out of the 65 commercially available meat products, 10 showed the signs of adulteration.

3. In PCR-SSCP system and using universal primer pairs, 16 Hungarian freshwater fish species were simultaneously distinguished from each other on the basis of the conformational differences of their single strand DNA on polyacrylamide gel. These 16 fish species were the following: rainbow trout, catfish, African catfish, common carp, Prussian carp, silver bream, common bream, rudd, bleak, roach, asp, stone moroko, tench, zander and northern pike. Using fluorescent marking of the applied universal primer pairs, catfish, African catfish, black bullhead, common carp, Prussian carp and rainbow trout species were studied in capillary

electrophoresis system. Out of the 6 species investigated, catfish and African catfish did not show any differences, while the other species exhibited different patterns.

9. Practical utilisation of the results

The PCR-SSCP method may be a relatively low-cost and time-efficient alternative for the further species identification investigations. The planned universal primers can be widely used for poultry, mammal and fish species, as well, decreasing on the costs of the method. However, this study covered the majority of the livestock, the universal primer pairs may be tested for the sequences of other species with a bioinformatic approach to determine whether the conservative regions identified for the species involved in this study are found in other species, as well, and whether it gives rise to the application of the method in a broader region in the consumer protection.

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11. List of publications

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Registry number: DEENK/266/2015.PL
Subject: Ph.D. List of Publications

Candidate: Ádám Csikós
Neptun ID: EN4NXN
Doctoral School: Doctoral School of Animal Husbandry
MTMT ID: 10047736

List of publications related to the dissertation

Hungarian scientific article(s) in Hungarian journal(s) (2)

1. **Csikós Á.**, Simon Á., Homonai K., Gulyás G., Tisza Á., Tamás A., Reglődi D., Jávor A., Czeglédi L.: Polimorfizmus keresése a szarvasmarha hipofízis adenilát-cikláz aktiváló polipeptid 5. exonjában.
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2. **Csikós Á.**, Simon Á., Tisza Á., Gulyás G., Jávor A., Czeglédi L.: PCR-TTGE módszer alkalmazása DNS mutációk kimutatására.
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Foreign language scientific article(s) in international journal(s) (2)

4. Tisza, Á., **Csikós, Á.**, Simon, Á., Gulyás, G., Jávor, A., Czeglédi, L.: Identification of poultry species using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and capillary electrophoresis-single strand conformation polymorphism (CE-SSCP) methods.
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