

Identification of Bovine, Pig and Duck Meat Species in Mixtures and in Meat Products on the Basis of the mtDNA Cytochrome Oxidase Subunit I (*COI*) Gene Sequence

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The aim of this study was to develop a method using PCR and self-designed primers on the basis of the mtDNA cytochrome oxidase subunit I (*COI*) gene sequence to enable direct identification of the meat of three species of animals, *i.e.* bovines, pigs and ducks, in the single type sample, in meat mixtures and meat products. The mixtures comprised up to six meat species including apart from beef, pork and duck also chicken, turkey and goose meat. The obtained results indicate the possibility of qualitative identification of the aforementioned meat species in all types of investigated food products. The maximum length of PCR products did not exceed 300 bp, which was supposed to favour the amplification of DNA from meat products which are usually thermally processed and/or exposed to high pressure. PCR primers hybridised selectively with bovine, pig and duck DNA, showing total species specificity.

INTRODUCTION

The analytical methods based on DNA analysis belong to the group of the most appreciated methods in research on the verification of the authenticity of meat species. It results from the specific character of the structure of deoxyribonucleic acid (DNA) particles and the possibility of using the information included in them [Karabasanavar *et al.*, 2011].

The researchers concerned with species identification pay increasing attention to the cytochrome oxidase subunit I (*COI*) gene. In 2003, the gene was selected for the identification of global biodiversity of eukaryotic organisms in the DNA barcoding method [Herbert *et al.*, 2003].

Wong & Hanner [2008] used the barcoding method in their studies to prove the adulteration of the fish known as red snapper (*Lutjanus campechanus*) with other species of fish or the adulteration of white tuna meat with tilapia meat. The method which uses the 'biological tag' also proved to be effective in differentiating bovine meat (*Bos taurus*) and southern reedbeak meat (*Redunca arundinum*) [Dalton & Kotze, 2011]. Universal primers were used to amplify a fragment of the bovine and chicken *COI* genes, thus indicating the usefulness of this method for the differentiation of meat species [Dawnay *et al.*, 2007]. However, it is noteworthy that the barcoding method, which uses DNA sequencing, is more suitable for the analysis of samples with individual meat types rather than complex mixtures. In the latter case, when, *e.g.*

Sanger sequencing is used, it is necessary to conduct additional procedures, *i.e.* to clone PCR products [Pereira *et al.*, 2008].

Feligini *et al.* [2005, 2007] used bovine-specific PCR primers, which were designed on the basis of the *COI* gene sequence, and made a qualitative and quantitative identification of cow's DNA in mozzarella cheese.

Haider *et al.* [2012] used *COI* sequences and seven restrictive endonucleases to identify raw bovine, chicken, turkey, sheep, pig, camel and donkey meat with the method assessing the restriction fragments length polymorphism (PCR-RFLP). In spite of the fact that the method using the *COI* gene enabled the differentiation of even such closely related species as the cow and buffalo or the chicken and turkey, its application may sometimes lead to false results. This may be related to the possibility of incomplete hydrolysis of restriction sites because of the enzymes used for investigations or to the occurrence of mutations in restriction sites within the species under investigation [Pereira *et al.*, 2008; Ballin *et al.*, 2009; Gil, 2007].

Furthermore, it is not always possible to obtain a long PCR product (700 bp) with recognisable sites to restriction enzymes, as the PCR-RFLP technique requires, from the DNA of meat products which were heated [Fajardo *et al.*, 2006, 2010; Eaqub *et al.*, 2012].

Therefore, the aim of our work was to create three species-specific primer pairs based on the *COI* gene structure. These primers would enable direct identification of meat from three species of animals, *i.e.* bovines, pigs and ducks, in the single meat sample, in meat mixtures and meat products. The study included the meat from three species of animals, which belong

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to the group of animals the most often slaughtered on an industrial scale in Poland [Statistical Yearbook of Agriculture, 2013].

MATERIALS AND METHODS

Meat samples

Apart from bovine meat (*Bos taurus*), pig meat (*Sus scrofa f. domestica*) and duck meat (*Anas platyrhynchos*), chicken meat (*Gallus gallus*), turkey meat (*Meleagris gallopavo*) and goose meat (*Anser anser*) samples were also collected. As far as the bovine and pig meat is concerned, the samples were collected from the longest lumbar muscle (*m. longissimus lumborum*), whereas poultry meat samples were collected from the pectoral muscle (*m. pectoralis*). On collection, the research material was placed in sterile test tubes and stored at a temperature of -80°C until DNA was isolated.

Meat mixtures

Twenty three meat mixtures were prepared for the research. Twenty one of them consisted of two meat components (Table 1).

The other two meat mixtures had the following qualitative and quantitative compositions: 20% beef (B), 20% pork (S), 20% chicken meat (C), 20% turkey meat (T), 10% duck meat (D), 10% goose meat (G) the first one, and 25% B, 25% S, 25% C, 25% T the second one.

Meat products

The research also included an analysis of seventeen meat products purchased in chain stores. The following commercial meat products were investigated: medium minced, smoked, the Polish raw sausages (sample 1 and 16 – pork);

cut, smoked and cooked frankfurters (sample 2, 5, 6 – pork), (sample 3 – turkey meat, pork and cheese addition), (sample 4 – pork and milk protein addition), (sample 8 – chicken meat, pork fat), (sample 9 – pork, chicken, turkey meat); raw, medium minced and smoked Frankfurter sausages (sample 7 – pork); medium minced, smoked and roast sausage (sample 10 – pork, beef); coarsely minced, smoked, cooked, slightly dried 'Krakow' sausage (sample 11 – pork, beef); finely minced, raw, smoked, slightly dried salami (sample 12, 13 – beef, pork, pork fat); sterilised canned product – duck terrine (sample 14 – duck fat and meat, poultry liver) and goose rillettes (sample 15 – goose meat, pork, pork fat); medium minced, smoked, cooked, slightly dried 'Kabanos' sausage (sample 17 – goose, pork, turkey meat).

DNA isolation

DNA was isolated from raw animal tissue, mixtures and meat products by means of the *PureLink™ Genomic DNA kit* (Invitrogen, Carlsbad, CA, USA), according to the methodology provided by the manufacturer. The DNA was stored for a short time at a temperature of +4°C and then frozen at -20°C.

Primers design

Species-specific primers for the identification of bovine, pig and duck meat were designed on the basis of nucleotide sequences of the *COI* gene acquired from GenBank from the NCBI base (*National Center for Biotechnology Information*, Bethesda, MD, USA) and Primer3 program application (Table 2). The primers were synthesised with TIB MOLBIOL (*Syntheselabor GmbH*, Berlin, Germany).

Species-specific PCR

The total volume of the mixture for the PCR was 20 µL. Each time it contained 40 ng DNA, 2 µL 10 × concentrated buffer for the PCR (Sigma, Saint Louis, MO, USA), 1 µmol/L of each pair of primers (TIB MOLBIOL), 0.25 mmol/L dNTP (Sigma) and 0.03 U of Taq DNA polymerase (Sigma).

As far as the primers for the identification of duck meat are concerned, reagents provided by the Invitrogen company were used in the study. The PCR sample contained 2 µL 10 × concentrated buffer for the PCR without MgCl₂ (Invitrogen), 5 mmol/L MgCl₂ (Invitrogen), 1 µmol/L of each pair of primers (TIB MOLBIOL), 0.25 mmol/L dNTP (Sigma) and 0.03 U of Taq DNA polymerase (Sigma).

TABLE 1. The qualitative and quantitative composition of meat mixtures.

S:B	B:S	C:D	T:S	D:S	G:C
S:C	B:C	C:B	T:C	D:T	–
S:T	B:G	C:S	–	–	–
S:D	B:T	C:T	–	–	–
S:G	B/S	C/S	–	–	–
–	–	C/B	–	–	–

Individual types of meat are abbreviated with the following letters: S – pork, B – beef, C – chicken meat, T – turkey meat, D – duck meat, G – goose meat. The meat proportions were 9:1 (abbreviations separated with „:”) or 1:1 (abbreviations separated with „/”).

TABLE 2. The pairs of PCR primers designed on the basis of the cytochrome oxidase subunit I gene fragment.

Species	Primer name	Accession number	Primer sequence 5' – 3'	Amplified fragment	Size of PCR product (bp)
Bovine	BTCOIF11 BTCOIR11	AF492351	GAACTCTGCTCGGAGACGAC ^a GGTACACGGTTCAGCCTGTT	134–388	255
Pig	SSCOIF11 SSCOIR11	NC_012095.1	GGAGCAGTGTTCGCCATTAT TTCTCGTTTGTATGCGAATG	1150–1443	294
Duck	APCOIF11 APCOIR11	L22480	TAATTGGCACAGCACTCAGC TTATCAGGGGGACCAATCAG	86–262	177

^a The *forward* primer, which is specific to the DNA of bovine's with identical sequence was earlier applied by Feligini [2005, 2007].

The polymerase chain reaction was conducted by means of a peqSTAR thermocycler (PEQLAB, Erlangen, Germany) using the following amplification profile: initial denaturation at 95°C for 9 min, followed by 30 cycles each of denaturation at 94°C for 1 min, annealing at 60°C (for bovine), at 59°C (for pig), at 71°C (for duck) for 1 min, extension at 72°C for 3 min. The final extension was at 72°C for 10 min.

The obtained PCR products were separated in 1% agarose gel for 45 min at a voltage of 100 V (Power PAC 300, Bio-RAD, Hercules, CA, USA). Sizes of DNA amplification products were identified using a *PCR 100 bp Low Ladder* (Sigma) size marker. The results of the separation were observed by means of a G:Box transilluminator (Syngene, Frederik, MD, USA).

PCR product sequencing

The PCR products were sequenced by means of the cyclic method with the use of capillary electrophoresis and a MegaBACE apparatus (General Electric, USA). Before sequencing, the PCR products were cleaned with a 7.5 mol/L ammonium acetate and ethanol solution from the remains of the polymerase, buffer, primers and nucleotides. The obtained DNA sequences were analysed by application of *ChromasPro* software (Technelysium Pty Ltd) and then their homology was compared with the reference sequences using the BLAST program (NCBI).

RESULTS AND DISCUSSION

The first step of the study involved tests of PCR primers with DNA extracted from single raw meat samples (beef, pork, duck, chicken, turkey and goose meat). All the primers designed on the basis of the *COI* gene were proved to exhibit specificity for the DNA from three species of animals, *i.e.* bovines, pigs and ducks, giving PCR products with intended size only with DNA for which amplification was created (data not shown).

The fact that the designed pairs of primers amplified the species-specific fragment of the *COI* gene was confirmed by the result of sequencing of the obtained PCR products. The sequencing of nucleic acids enables determination of the primary DNA structure and provides an answer to the question which gene fragment was amplified in the PCR [Kalak *et al.*, 2004; Sambrook & Russell, 2011].

The obtained DNA sequences were analysed by means of the *ChromasPro* program. Then they were compared with the reference sequences for individual animal species on the basis of which the PCR primers were designed. The results of the analysis confirmed the fact that all the three pairs of PCR primers, *i.e.* those designed for the amplification of bovine, pig and duck DNA, hybridised with the fragment of the *COI* gene.

The comparison of the sequences obtained from the PCR product which was formed as a result of the amplification of the DNA with the primers for bovines (BTKOIF11 and BT-COIR11) and pigs (SSCOIF11 and SSCOIR11) with the reference sequences proved their identity in 100%.

The comparison of the nucleotide sequence of the PCR product formed as a result of the use of primers for the duck

(APCOIF11 and APCOIR11) with reference sequences proved 98% similarity between them. The absence of 100% compatibility between the sequences obtained from the sequencing of the duck PCR products with reference sequences may have been related to certain intraspecies differences. It is most likely that the *COI* gene sequences which were used to design the duck primers came from different duck breeds than those from which the research material was acquired.

Next, the PCR primers were tested on raw meat mixtures. The presence of DNA from different meat species in one sample may disturb the formation of PCR products. The research conducted by Iwobi *et al.* [2011] proved that it was difficult to detect the chicken or turkey meat in a sample where its content was lower than 0.5% due to the dominance of pork and beef. On the other hand in the studies carried out by Partis *et al.* [2000], PCR primers preferred to amplify the DNA sequence a small amount of which was present in the mixture. This observation referred to the primers used in the PCR-RFLP technique, where one pair favoured the amplification of the pig DNA, only 1% of which was present in the mixture, whereas the other one preferred the amplification of the bovine DNA over other species. Also Dawnay *et al.* [2007] observed that it was impossible to identify the bovine or chicken DNA, which had considerable quantitative preponderance in the mixture (10:1) over the human DNA.

The results of the analysis revealed that all the three pairs of PCR primers designed to amplify the bovine, pig and duck DNA correctly identified these meat species in the meat mixtures. Each of the pairs of primers enabled the amplification of the DNA from the meat of which 10% could be found in the mixture. This reaction was also observed for the duck primers in the six-component mixture (data not shown).

The last and most demanding stage of the research was to test the designed set of primers and the established PCR thermal profile on the DNA acquired from meat products. The species identification based on nucleotide sequences always requires that an appropriate quality and quantity of DNA specimens should be acquired. It is particularly difficult in the case of processed food. During the extraction of DNA from meat products to specimens such compounds as Maillard reaction products, milk proteins, fat, glycogen, collagen, iron, cobalt, *etc.* may also be transferred. These compounds inhibit DNA amplification and may significantly affect the result of the analysis [Cammá *et al.*, 2012; Wilson, 1997]. Additionally, apart from the target DNA to be investigated, the sample under analysis may also contain the DNA of other origin, *e.g.* from bacteria, plants, animals [Teletchea *et al.*, 2005]. Besides that, some enzymes, *e.g.* those which are present in soy specimens, may cause DNA degradation [Musto, 2011]. That is why it is necessary to remember that the absence of the product of DNA amplification after the PCR may be related not only to the insufficient amount or absence of DNA from the sample, but also to the inhibiting effect of contaminants in the specimen under analysis. Therefore, it is a key element of the experiment to remove all PCR inhibitors [Teletchea *et al.*, 2005; Musto, 2011].

The outcome of amplification is significantly influenced not only by the DNA isolation procedure adequate to the biological material under analysis but also by the technological

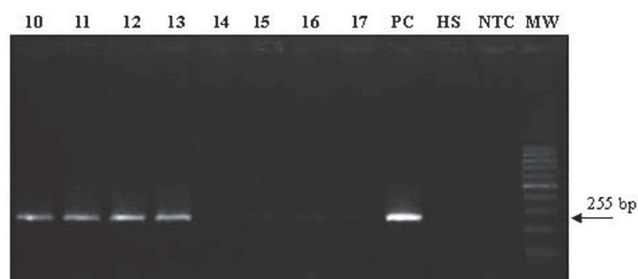


FIGURE 1. The electrophoretic separation of PCR products in 1% agarose gel. A polymerase chain reaction was conducted with the bovine primers: BTCOIF11 and BTCOIR11 with the DNA isolated from meat products. Individual lanes were marked with the numbers which correspond to particular sample – see Materials and Methods chapter. Letter abbreviations: PC – positive control sample, HS – human DNA, NTC – negative control sample, MW – size marker.

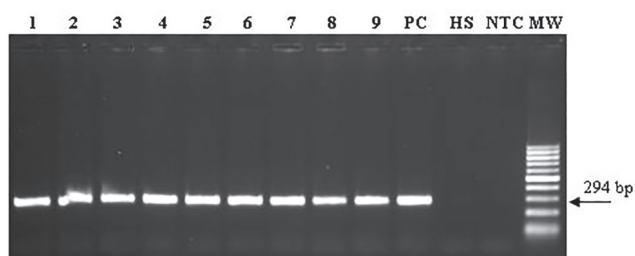


FIGURE 2. The electrophoretic separation of PCR products in 1% agarose gel. A polymerase chain reaction was conducted with the pig primers: SSCOIF11 and SSCOIR11 with the DNA isolated from meat products. Description of particular lines – see Figure 1.

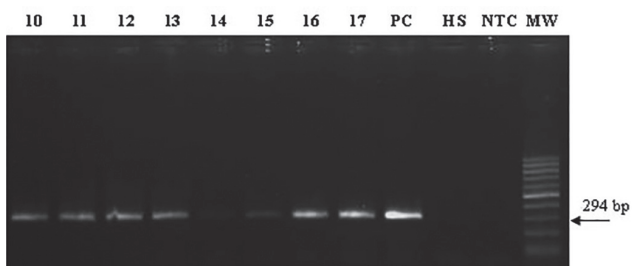


FIGURE 3. The electrophoretic separation of PCR products in 1% agarose gel. A polymerase chain reaction was conducted with the pig primers: SSCOIF11 and SSCOIR11 with the DNA isolated from meat products. Description of particular lines – see Figure 1.

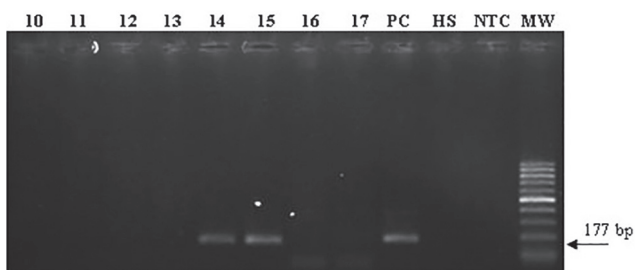


FIGURE 4. The electrophoretic separation of PCR products in 1% agarose gel. A polymerase chain reaction was conducted with the duck primers: APCOIF11 and APCOIR11 with the DNA isolated from meat products. Description of particular lines – see Figure 1.

process applied in the production of cold cuts. DNA may be partly degraded as a result of the standard procedures applied in the manufacturing process of meat products (e.g. boiling, sterilisation). In some cases, the fragmentation of DNA may lead to the development of a background or it may even make it impossible for a PCR product to be formed, which makes the analysis more difficult [Musto, 2011].

Also, certain environmental factors (low pH, UV radiation, humidity) may cause a chemical modification of the DNA particle, resulting in its fragmentation or in the formation of PCR artefacts. In consequence, this may cause the appearance of misleading results [Teletchea *et al.*, 2005].

This part of the research included analysis of the ability of the designed PCR primers to identify the bovine, pig and duck DNA in 17 meat products. Some products contained considerable amounts of fat and other additives, which are routinely applied in the technological process, according to the rules of good manufacturing practices. In pâtés, the content of fat usually amounted to about 20%, whereas in finely minced products, such as Vienna sausages, the fat content reached about 35–40%. It is noteworthy that in the production process the meat underwent various technological treatments, such as salting, curing, smoking, cooking, pre-drying or even sterilisation. The PCR which was conducted with the use of bovine-specific primers with the DNA acquired from 17 meat products showed that the bovine DNA could be found in samples 10–13, which was in agreement with the ingredients declared by manufacturers on product labels (Figure 1).

The analysis of meat products for pork content proved that this type of meat could be found in all of the samples under investigation, *i.e.* 1–17, except sample number 14. As a rule, it was in agreement with the manufacturers' statements (Figures 2, 3). According to the manufacturer's declaration, there was no pig meat in sample '8', but the list of ingredients included pig fat, which may have contained elements of the pig muscle tissue. This *status quo* probably affected the result.

A pair of primers specific to the duck DNA proved that the DNA of this animal was present in samples '14' and '15' (Figure 4). Whereas the presence of the duck DNA in sample '14' is justified, the presence of this DNA in sample '15' proves that it was added deliberately, which means that it was used as a substitute or to adulterate the product. As was proved in earlier stages of the research, *i.e.* during the reactions with the DNA from raw duck meat and raw meat mixtures, the primers designed for the duck were specific only to the duck DNA. Thus, a cross-reaction could not have taken place here, but part of the goose meat was deliberately replaced with duck meat.

In this research, the primers hybridising with the bovine, pig and duck DNA proved full species specificity. The obtained results also point to the fact that a PCR product can be obtained not only from raw products but also from the products which underwent cooking, roasting or even sterilisation.

Apart from that, the selected DNA isolation method resulted in obtaining the appropriate quality of DNA specimens, which were free from the contaminants which may have made it difficult to obtain reliable results.

The usefulness of the *COI* gene and scientists' interest in using its sequences for the identification of animal DNA is proved by the research by Natonek-Wiśniewska *et al.* [2013]. Their findings indicated the feasibility of detecting 0.09% of the pig DNA in feeds made from both vegetable and animal raw materials by means of PCR primers designed on the basis of the *COI* gene sequence.

To sum up, the results of this study and the findings of other authors' studies [Herbert *et al.*, 2003; Wong & Hanner, 2008; Dalton & Kotze, 2011; Dawnay *et al.*, 2007; Pereira *et al.*, 2008; Feligini *et al.*, 2005, 2007; Haider *et al.*, 2012; Natonek-Wiśniewska *et al.*, 2013] give grounds for the conclusion that *COI* gene sequences are very useful for analyses related with the identification of species in the meat of slaughter animals, conducted by means of the methods of molecular biology.

CONCLUSIONS

The findings presented in this study confirmed the fact that it is possible to identify the meat of three species of animals, *i.e.* bovines, pigs, and ducks, on the basis of the *COI* gene sequence. All the three sets of designed primers showed full specificity for the bovine, pig and duck DNA sequences, respectively. It was possible to conduct species-specific amplification not only from the samples where DNA came from raw meat but also from those where it came from mixtures and processed meat products. The developed method can be a useful tool for meat species detection tests. This study needs to be continued to estimate sensitivity level, *i.e.* the lowest amount of meat species which can be identified in meat products by application of self-designed PCR primers.

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