SPECIES DIFFERENTIATION OF CHICKEN AND DUCK MEAT BY ANALYSIS OF THE CYTOCHROME OXIDASE SUBUNIT I AND APPLICATION PCR TECHNIQUE

Anita Spychaj[1*], Marlena Szalata[2, 3], Ryszard Słomski[2, 3], and Edward Pospiech[1, 4]

[1]Institute of Meat Technology, Poznań University of Life Sciences, Wojska Polskiego 31, 60 - 624 Poznań, Poland

[2]Department of Biochemistry and Biotechnology, Poznań University of Life Sciences, Wołyńska 35, 60 - 637 Poznań, Poland

[3]Institute of Human Genetics, Polish Academy of Sciences, Strzeszyńska 32, 60 – 479 Poznań, Poland

[4]Institute of Agricultural and Food Biotechnology, Głogowska 239, 60 – 111 Poznań, Poland

[*]Corresponding author (phone: +48-61-848-7505; fax: +48-61-848-7254; e-mail: aspychaj@up.poznan.pl)

***Abstract*—Control of animal species composition of meat products became more and more necessary. The reason for that is increasing number of cases of food adulteration or unintended its contamination as result of disobeying GMP and GHP rules. To reduce such situations there is need to develop reliable methods for control meat products composition. The aim of the presented study was to elaborate new method to detect chicken and duck meat by using PCR technique. As a molecular target the cytochrome oxidase subunit I (mtDNA) was chosen. Nucleotide sequences for primers designing were obtained from GenBank (NCBI). To confirm primers specificity and exclude probability of cross reactions, chicken, duck but also turkey, goose, cattle and pig DNA was isolated and used in PCR. The obtained data indicate that designed primers for chicken and duck were species specific. Conducted sequencing of PCR products confirmed that these two primers sets amplified fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene. The obtained results indicate that the identification of chicken and duck raw meat is possible by application of PCR primers designed on the base of the cytochrome oxidase subunit I.**

***Index Terms*— mitochondrial DNA, PCR, species identification.**

I. INTRODUCTION

Nowadays more and more aware of their rights consumers, require honest information, concerning origin and composition of food products. Customer should know about species composition purchased meat products. It is important, first of all, because of health but also economical, dietetic and even religious reasons. Unfortunately, there are some cases of food adulteration or unintended contamination resulting from disobeying GMP and GHP rules. As shows the investigations concerning the authenticity of meat products carried out by Rao and Hsiesh (2007) in the United States, 62% samples of maturing sausages were contaminated with other meat species: 36% with two species and 2% with three species. In experiments conducted by Pascoal, Prado, Castro, Cepeda and Barros-Velázquez (2004) on 50 industrially manufactured meat articles, it was demonstrated that out of 30 products declared to have been manufactured from one meat species, ten contained additions of meat derived from other species. In the case of the remaining 20 products whose labels indicated the presence in them of at least two meat species, five articles did not contain one of the declared species.

Therefore, there is the need to develop dependable methods for reliable meat species identification. For species differentiation methods based on isotope analysis, immunological, chromatographic, electrophoretic and genetic analyses are applied. Genetic methods give great possibilities in determining authenticity of meat products, and this fact is strictly connected with nucleic acids characteristic. DNA is more resistant to high temperature and pressure than proteins. Such properties cause that DNA can be used to identify meat species also in processed meat products submitted to technological treatments (Hird, Chisholm and Brown, 2005).

In the presented study a fragment of the cytochrome oxidase subunit I (mtDNA) was applied. Mitochondrial DNA is widely use for species identification, due to presence in many copies in cells. It has high rate of mutations and diversity in its sequence. Such conditions allow differentiation even closely related species (Unseld, Beyermann and Brandt, 1995), (Pereira, Carneiro and Amorim, 2008). A fragment of the cytochrome oxidase subunit I was applied in past in experiments connected with detection cow's milk in water buffalo's milk cheeses (Feligini, Bonizzi, Curik, Parma, Greppi and Enne, 2005, Feligini, Alim, Bonizzi, Enne and Aleandri, 2007).

The aim of the presented study was to distinguish and identify chicken and duck meat. Duck meat as a more expensive, can be replaced by dishonest food manufactures, by cheaper chicken meat. To detect or prevent such unfair practices it was intended to develop method based on amplification a fragment of the cytochrome oxidase subunit I using species specific primers and employing PCR technique.

II. MATERIALS AND METHODS

A. DNA isolation

DNA from raw meat of six animal species i.e. chicken (*Gallus Gallus*), duck (*Anas platyrhynchos f. domestica*), turkey (*Meleagris gallopavo*), goose (*Anser anser f. domestica*), cattle (*Bos taurus*) and pig (*Sus scrofa f. domestica*) was studied. It was isolated using of proteinase K method. For this purpose 50-150 mg of meat sample was placed into sterile tubes in 800 µl of solution consisted of 600 µl SE buffer (75 mM NaCl, 1 mM Na_2EDTA, pH 8,0), 100 µl 10% SDS and 20 µl proteinase K (10 mg/ml). The content of the tube was mixed and incubated at 55^{o}C overnight. Next 800 µl mixture of phenol, chloroform and isoamyl alcohol in ratio 25 : 24 : 1 respectively was added and centrifugation was performed at 13000 rpm for 10 min. at 4^{o}C. To the obtained supernatant 800 µl of 2–propanol was added and after gentle mixing, the samples were centrifuged again at 13000 rpm for 10 min. After supernatant removing, DNA was washed twice with 500 µl of 75% ethanol. Dried DNA pellet was resuspended in 100 µl of sterile water. The nucleic acid concentration and purity were determined by measuring absorption at 260 nm and 280 nm using Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA was stored at –18^{o}C until further analysis.

B. Primers designing

Species specific primers for detection of chicken (GGCOIF and GGCOIR) and duck meat (APCOIF and APCOIR) were designed on a base of nucleotide sequences for a fragment of the cytochrome oxidase subunits I (COI) obtained from GenBank (National Center for Biotechnology Information (NCBI), Bethesda, MD, USA). Primer sets (table 1) were prepared with the help of the program located at http://biotools.umassmed.edu/bioapps/primer3_www.cgi website and synthesized by TIB MOLBIOL (Syntheselabor GmbH, Berlin, Germany).

Table 1.

Primer name	Sequence (5’ – 3’)	PCR product size (bp)	Accession number
GGCOIF GGCOIR	GGCGCATCAGTAGACCTAGC CAGCTGCTAGGACGGGTAAG	196	AP 003580
APCOIF APCOIR	CCCCATAGTCCACGCTATTG TCGAAGCCCGTCTGTCTAGT	192	L 22480

C. PCR amplification

PCR was performed in total volume of 20 µl. The reaction mixture contained 100 ng of DNA, 10x PCR Gold Buffer (Applied Biosystem, Foster City, CA, USA), 2.5 mM $MgCl_2$ (Applied Biosystem, Foster City, CA, USA), 1 µM each primers (TIB MOLBIOL, Syntheselabor GmbH, Berlin, Germany), 0.25 mM dNTP (Sigma, Saint Louis, MO, USA) and 0.125 units of polymerase Taq (Applied Biosystem, Foster City, CA, USA). Following conditions for PCR were used: initial denaturation at 95ºC for 9 min, denaturation at 94ºC for 60 s, annealing at 56ºC for 60 s, extension at 72ºC for 180 s. For each pair of primers, 30 cycles of amplification were carried out followed by final extension for 5 min at 72ºC. PCR was performed using peqSTAR thermocycler (PEQLAB, Erlangen, Germany).

D. Electrophoresis

The obtained PCR products were separated in 1,5% agarose gel (13 x 15 cm) with 0,003% ethidium bromide for 50 min at 80 V (Power PAC 300, Bio – RAD, Hercules, CA, USA). To identify PCR product size, the size marker 100 bp Low Ladder was used (Sigma, Saint Louis, MO, USA), and the results were observed on transilluminator G – Box (Syngene, Frederik, MD, USA).

E. PCR products sequencing

PCR products of the cytochrome oxidase subunit I both, for chicken and duck were cloned into plasmid vector pGEM-T Easy (Promega Corp., Madison, WI, USA). To make cloning easer the ability of polymerase Taq to join adenine to 3’ ends of PCR products was exploited. Thanks to this it was possible to use plasmid vector with thymine on its 5’ ends. In this way the efficiency of ligation was improved. PCR products were ligated into plasmid vector with the help of DNA T4 ligase. The products of ligation were inserted to competent cells of *E. coli* of XL1Blue strain by transformation method. Confirmation of recombination was performed by colony PCR. Inserted fragments of DNA were amplified using pGEMF (5’-CGACTCACTATAGGGCGAAT-3’) and pGEMR (5’-GGTGACACTATAGAATACTCAAGC-3’) primers, specific for flanking region of multiple cloning site. After

preliminary clones' selection by using colony PCR method, plasmid DNA was isolated using the Qiagen Maxi kit (Qiagen, Inc., Valencia, CA, USA) from chosen bacteria colonies and sequenced by cyclic method with the aid of primers M 13F and M 13R. The obtained mtDNA sequences (MEGA Bace Sequencer, GE Healthcare, Buckinghamshire, UK) were analyzed by using ChromasPro program. Next the sequences were compared with reference sequence DNA available in GenBank using BLAST program (Basic Local Alignment Search Tool) located at NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) website.

III. RESULTS AND DISCUSSION

In the presented study fragment of the cytochrome oxidase subunit I (mtDNA) was employed to design species specific primers. These primers were used for differentiation chicken and duck meat from others species by application PCR technique. To compare nucleotide sequences of the cytochrome oxidase subunit I for chicken (AP 003580) and duck (L 22480) BLAST (NCBI) was applied. The obtained results indicated at lack of similarities between mentioned species and other which can be use for meat products manufacture. Species specific primers were designed with a help of computer program located at http://biotools.umassmed.edu/bioapps/primer3_www.cgi.

Primers pairs were tested on DNA isolated from raw meat of six animal species i.e. chicken, duck, goose, cattle and pig by application PCR technique. The obtained data indicate that primers designed on the basis of the cytochrome oxidase subunit I are species specific. Primers set designed to detect chicken meat (GGCOIF and GGCOIR) yielded 196 bp PCR products in reaction with chicken DNA, but did not work with duck, turkey, goose, cattle and pig DNA (Fig. 1). Similar situation was in case of duck primers (APCOIF and APCOIR). PCR product of duck DNA comprised 192 bp and any cross reaction with chicken, turkey, goose, cattle and pig DNA was observed (Fig. 2).

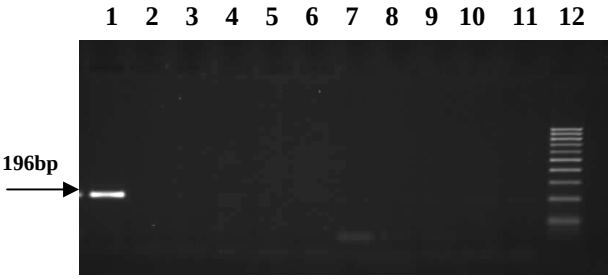

Fig. 1. Separation of PCR products in 1,5% agarose gel. Lane 1, chicken; lanes 2 – 3, cattle; lanes 4 – 5, pig; lanes 6 – 7, turkey; lanes 8 – 9, goose; lanes 10 – 11, duck; lane 12, PCR 100 bp Low Ladder (Sigma, Saint Louis, MO, USA). DNA was amplified with chicken primers: GGCOIF and GGCOIR.

Fig. 2. Separation of PCR products in 1,5% agarose gel. Lane 1, duck; lanes 2 – 3, chicken; lanes 4 – 5, cattle; lanes 6 – 7, pig; lanes 8 – 9, turkey; lanes 10 – 11, goose; lane 12, PCR 100 bp Low Ladder (Sigma, Saint Louis, MO, USA). DNA was amplified with duck primers: APCOIF and APCOIR.

To confirm that designed primers sets amplified fragment of the cytochrome oxidase subunit I, the sequencing of DNA amplicons was carried out. The obtained data indicate that designed species specific primers amplified fragment of the cytochrome oxidase subunit I. Comparative analysis for the sequence of chicken's clone 19 with reference sequence of mitochondrial DNA to the cytochrome oxidase subunit I (NW_001487649.1) was carried out with the aid of computer program BLAST (NCBI). The obtained results demonstrated 99% similarity between compared sequences. The difference concerned replacement nucleotide at the position 3070 T>C. Comparison of nucleotide sequence of duck's clone 31 with reference sequence of the cytochrome oxidase for duck (L22480.1) indicated 99% similarity between them. The difference concerned replacement nucleotide at the position 287 T >C.

It seems to be interesting to continue this study to develop other species specific primers sets for identification other species e.g. turkey, goose, cattle, pig etc. It would be advisable to verify its practical application in case of raw and heat treated meat mixtures.

IV. CONCLUSION

The obtained data indicate that there is a possibility to identify chicken and duck raw meat by PCR application. Using primers designed for a fragment the cytochrome oxidase subunit I. Created primers showed species specificity. Chicken and duck primers sets amplified chicken and duck DNA respectively, and cross-reactions with others analyzed species were not observed.

ACKNOWLEDGEMENT

This study was supported by Grant N N312 205636 from Polish Ministry of Science and Higher Education.

REFERENCES

Feligini, M., Bonizzi, N., Curik, V. C., Parma, P., Greppi, G. F., & Enne, G. (2005). Detection of adulteration in Italian Mozzarella cheese using mitochondrial DNA templates as biomarkers. *Food Technology and Biotechnology*, 43 (1) 91 - 95.

Feligini, M., Alim, N., Bonizzi, I., Enne, G., & Aleandri, R. (2007). Detection of cow milk in water buffalo cheese by SYBR Green real – time PCR: sensitivity test on governing liquid samples. *Pakistan Journal of Nutrition*, 6 (1): 94 - 98.

Hird, H., Chisholm J., & Brown, J. (2005). The detection of commercial duck species in food using a single probe - multiple species – specific primer real – time PCR assay. *European Food Research and Technology*, 221, 559 - 563.

Pascoal A., Prado M. Castro J., Cepeda A., & Barros-Velázquez J. (2004). Survey of authenticity of meat species in food products subjected to different technological processes, by means of PCR-RFLP analysis. *European Food Research and Technology*, 218, 306 - 312.

Rao, Q., & Hsieh Y.-H. P. (2007). Evaluation of a commercial lateral flow feed test for rapid detection of beef and sheep content in raw and cooked meats. *Meat Science*, 76, 489 - 494.

Pereira, F., Carneiro, J., & Amorim, A. (2008). Identification of Species with DNA - Based Technology: Current Progress and Challenges. *Recent Patents on DNA & Gene Sequences* 2, 187 - 200.

Unseld, M., Beyermann, B., & Brandt R. (1995). Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *PCR Methods and Applications*, 4: 241 - 243.

WEB REFERENCES

http://biotools.umassmed.edu/bioapps/primer3_www.cgi

http://blast.ncbi.nlm.nih.gov/Blast.cgi

http://www.ncbi.nlm.nih.gov/nucleotide/