Animal Species Identification by PCR – RFLP of Cytochrome b

Tomáš Minarovič, Anna Trakovická, Alica Rafayová, Zuzana Lieskovská

Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76, Nitra, Slovakia

Abstract

An alternative DNA detection system is based on the polymerase chain reaction (PCR) amplification of a segment of the mitochondrial cytochrome b gene. Subsequent cleavage by a restriction enzymes gives rise to a specie-specific pattern on an agarose gel. We used five animal species (*Mustela vison*, *Mustela putorius furo*, *Sus scrofa domesticus*, *Oryctolagus cuninculus*, *Anser anser*). Length of PCR product was 359 bp and we used universal primers. Restriction fragment length polymorphism was analyzed by using the restriction endonuclease *Alu*I. Results of cleavage were visualized by using electrophoresis and UV transiluminator. Every animal specie has a unique combination of restriction fragments i.e. *Mustela vison* 81 bp, 109 bp and 169 bp, *Mustela putorius furo* 169 bp and 190 bp, *Sus scrofa domesticus* 115 bp and 244 bp, *Oryctolagus cunninculus* is not cleaved by *Alu*I so it has whole 359 bp fragment on agarose gel, *Anser anser* 130 bp and 229 bp. The results suggest that the method of PCR - RFLP is rapid and simple method for identification of species. PCR – RFLP can reliably identify chosen species. Application of genetic methods is very useful for breeding of livestock and protection of biodiversity.

Keywords: animal biodiversity, cytochrome b, mitochondrial DNA, PCR – RFLP,

1. Introduction

Animal species identification is very important especially in protection of animal biodiversity, veterinary diagnostics and for food samples control in food industry. Utilization of polymerase chain reaction of mitochondrial DNA (mtDNA) is modern method of animal species identification. Mitochondrial DNA is very useful for its multiple presences in cells. The most of animal mtDNA is coding 37 genes [1]. One of them is gene for cytochrome b (CytB). Cytochrome b is a component of respiratory chain complex III [2, 3]. Length of CytB gene is 1140 bp and has some stable sequences which were used for suggestion of universal primers and some variable sequences used for animal identification by PCR-RFLP method. We amplified 359 bp sequence of cytochrome b and cleaved it by restriction enzyme *Alu*I. After cleavage we could observe specific patterns for each animal specie used in this study. It is very important to choose right endonuclease for each group of chosen animal species. PCR-RFLP is accurate, cheap and quick method for identification. Many studies were published e.g. Lenstra et al. in 2001 [4] used 9 animal species (chicken, turkey, pig, cattle, water buffalo, sheep, goat, horse, human) and 6 restriction endonucleasis (*Alu*I, *Hae*III, *Hinf*I, *Mbo*I, *Rsa*I, *Taq*I) for their cytb PCR-RFLP identification. Pascoal et al. [5] in 2004 proved that PCR-RFLP method is rapid and easy-to-perform two step analytical approach to achieve qualitative meat species identification in raw and even in cooked food products containing one or more different species.

* Corresponding author: Tomáš Minarovič, Slovak University of Agriculture, Department of genetics and breeding biology, Tr. A. Hlinku 2, 949 76, Nitra, Slovakia,00421-376414824, Tomas.Minarovic@azet.sk

2. Materials and methods

We used for our study blood samples from five animal species (*Mustela vison* –American mink, *Mustela putorius furo* – Ferret, *Sus scrofa domesticus* – Domestic pig, *Oryctolagus cuninculus* – Rabbit, *Anser anser* – Goose). Blood from all animals were collected to the Eppendorf tube with anticoagulant acid citrate dextrose (ACD) solution in proportion to 5:1 (blood : ACD) and kept frozen for later determinations.

The DNA was extracted according to Miller et al. (1987) [6] from 300 µl of blood or by using commercial kit Nucleospin Blood (Macherey Nagel - Biotech).

PCR of 359 bp long sequence of cytochrome b was performed by using of primers designed as described Kocher et al. (1989) [7]. The PCR was prepared according to Gábor et al. (2009) [8]. The 25 µl reaction mix was prepared in an Eppendorf tube containing of 1 µl of template DNA (concentration 50 $ng.l^{-1}$), 0,2 µl of Taq DNA polymerase (Fermentas), 0,75 µl of CytB primers mix (3 pmol from each primer CytB FOR: 5′– CCA TCC AAC ATC TCA GCA TGA TGA AA – 3′ and CytB REV: 5′– GCC CCT CAG AAT GAT ATT TGT CCT CA - 3′), 0,5 µl of deoxynucleotide triphosphate (2 mM dNTP), 1,7 µl of 25 mM $MgCl_2$, 2,5 µl of 10 x reaction buffer $(NH_4)_2SO_4$ and 18,35 µl of H_2O. The thermal cycler Bio-rad C1000™ was programmed for 35 cycles of PCR. PCR started with 94 °C for 90 seconds. Each cycle included denaturation step for 10 seconds (94 °C), annealing step for 30 sec. (55 °C) and polymerization step for 40 sec. (72 °C). After 35 cycles followed 10 minutes of elongation (72 °C) and cooling down to 4 °C and storage. We used 2 % agarose gel and electrophoresis for checking of proper amplification of 359 bp CytB PCR fragment (figure 1).

The amplicons were digested with *Alu*I, restriction enzyme, for 8 hours (37 °C) resulting in a patterns that could identify and differentiate each of the above species. 25 µl of cleavage mix consisted from: 1 µl of *Alu*I restriction enzyme (Fermentas) (final concentration 10 $U.\mu l^{-1}$), 2 µl of 10x Tango Buffer (fin. conc. 1x), 8 µl of PCR product and 14 µl of H_2O. Digestion was stopped by 3,5 µl of Xylene cyanol FF. Digested fragments were visualized by electrophoresis on 2 % agarose gel at 130 V in 0,5x TBE for 30 minutes on a 10 µl portion of the amplified DNA fragments (rabbit 5 µl). The resulting gel was stained with ethidium bromide 83 pl/ml (5µl in 60 ml of gel). Visualization of fragments was carried out by using of UV transilluminator and photographed with an Olympus C 700.

3. Results and discussion

We used polymerase chain reaction (PCR) for amplifying 359 bp fragment of gene for cytochrome b and restriction fragment length polymorphism method (RFLP) for identifying of different animal species. We used primers from study of Kocher et. al. (1989) [7] and chose restriction endonuclease by NEBcutter V2.0 [9], CLC Main Workbench V5.5 and sequences from NCBI database. 359 bp long fragment was digested by restriction enzyme *Alu*I to specie specific patterns (table 1, scheme 1). Results of digesting were visualized by electrophoresis on 2 % agarose gel (figure 2). Each animal specie chosen for this study was digested by *Alu*I at different place so it could create different sequence and we were able to identify each animal specie. PCR product of rabbit (*Oryctolagus cuninculus*) did not have restriction place for *Alu*I, so we could see the whole 359 bp long fragment on agarose gel. In theoretical case of the same fragments of chosen species we recommend to pick different restriction enzyme for RFLP identification.

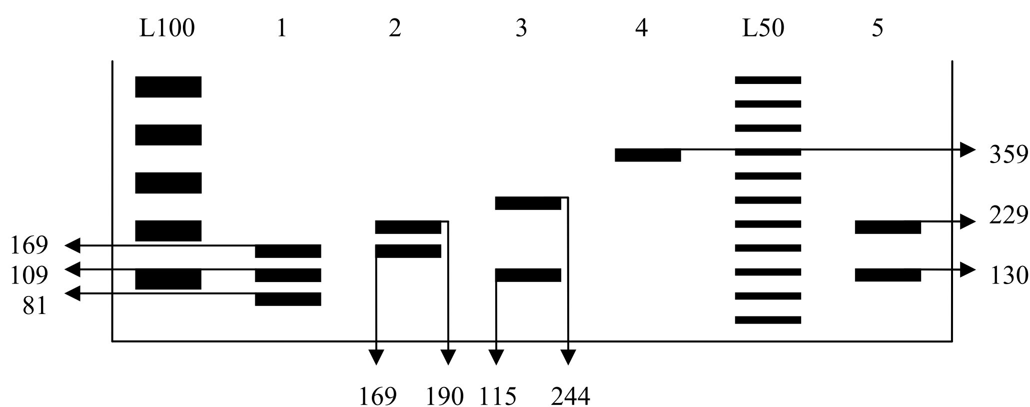
PCR – RFLP is often used for animal species identification. It is very quick, accurate and not too expensive method. Species identification of meat in meat products is essential from medical, ethical and economical point of view. PCR - RFLP method can be used for the analysis of genetically determined diseases in human and veterinary medicine. It can be also used to detection of oncogenes, in gene mapping, in phylogenetic analysis and for the study of association relations of candidate genes with performance indicators. PCR – RFLP is very susceptible to contamination so we recommend to take precautions against contamination.

Attention should be paid to the problem of possible undigested PCR fragments. In the present procedure this may lead to misinterpretation, because undigested animal PCR products could resemble a sample of human origin. However, due to the fact that PCR products do not posess methylated nucleotides which occur in plasmids

and genomic DNA, cleavage inhibition due to methylation cannot occur. Cleavage inhibition can be mediated by substances which originate from the DNA source and are transferred together with the template DNA into the PCR tube [10].

Table 1 Restriction fragments length after cleavage of 359 bp PCR product by *Alu*I

Animal specie	Restriction fragment
Mustela vison	81 bp, 109 bp, 169 bp
Mustela putorius furo	169 bp, 190 bp
Sus scrofa domesticus	115 bp, 244 bp,
Oryctolagus cuninculus	359 bp
Anser anser	130 bp, 229 bp



Scheme 1: Scheme of restriction fragments after cleavage of 359 bp PCR fragment of cytochrome b by restriction enzyme *Alu*I. L100 – molecular marker (100 bp), 1 – *Mustela vison*, 2 – *Mustela putorius furo*, 3 – *Sus scrofa domesticus*, 4 – *Oryctolagus cuninculus*, L50 – molecular marker (50 bp), 5 – *Anser anser*

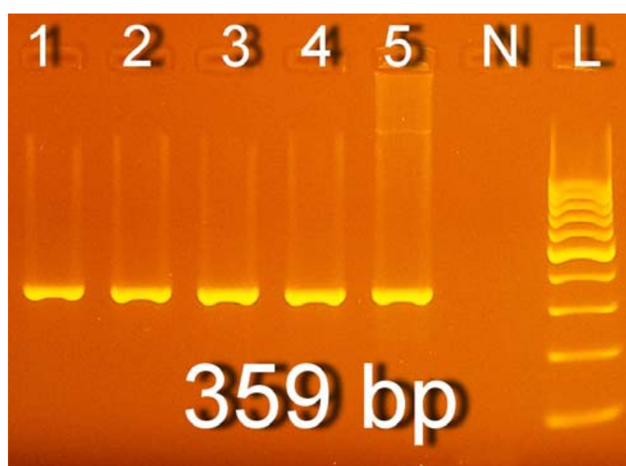


Figure 1. 359 bp PCR fragment of cytochrome b

1 – *Mustela vison*

2 – *Mustela putorius furo*

3 – *Sus scrofa domesticus*

4 – *Oryctolagus cuninculus*

5 – *Anser anser*

N – negative control of PCR

L – molecular marker (100 bp)

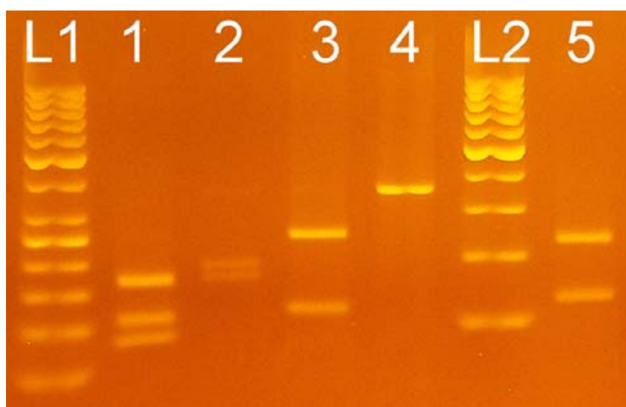


Figure 2. RFLP analysis. Specie specific restriction fragments. L1 – molecular marker (50 bp), 1 - *Mustela vison*, 2 - *Mustela putorius furo*, 3 - *Sus scrofa domesticus*, 4 - *Oryctolagus cuninculus*, L2 – molecular marker (100 bp), 5 - *Anser anser*

4. Conclusions

This study shows that species identification method based on the polymerase chain reaction (PCR) amplification of a segment of the mitochondrial cytochrome b gene, processed with restriction fragment length polymorphism (RFLP) method can be very reliable.

We amplified 359 bp PCR products, which were cleaved by a restriction enzyme. After digesting by an *Alu*I endonuclease we observed species-specific patterns on 2 % agarose gel. Every animal specie has a unique combination of restriction fragments i.e. *Mustela vison* (American mink) had 81 bp, 109 bp and 169 bp fragments, *Mustela putorius furo* (Ferret) had 169 bp and 190 bp fragmetns, *Sus scrofa domesticus* (Pig) had 115 bp and 244 bp restriction fragments, *Oryctolagus cunninculus* (Rabbit) wasn't cleaved by *Alu*I so it has whole 359 bp fragment, *Anser anser* (Goose) had 130 bp and 229 bp long restriction fragments. Results of digesting were visualized by using of electrophoresis and UV transiluminator.

The results suggest that the method of PCR - RFLP is rapid and simple method for identification of species. PCR – RFLP can reliably identify the chosen species. Application of genetic methods is very useful for animal breeding and protection of biodiversity.

Acknowledgements

The work was supported by VEGA project: "Genetic assessment of the meat production and quality in the special sectors of animal production." Project registration number: 1/0061/10

References

1. Avise, J. C., Molecular markers, natural history and evolution. 1994, Chapman & Hall, New York, N.Y.
2. Howell, N., Evolutionary conservation of protein regions in the protonmotive cytochrome b and their possible roles in redox catalysis. In: *J. Mol. Evol.* 29 (2), 1989. pp. 157–169. doi:10.1007/BF02100114. PMID 2509716.
3. ESposti, M. D., Crimi, M., Ghelli, A., Patarnello, T., Meyer, A., De Vries, S., Mitochondrial cytochrome b: evolution and structure of the protein. In: *Biochim. Biophys. Acta* 1143 (3), 1993. pp. 243–271. doi: 10.1016/0005-2728(93)90197 - N. PMID 8329437
4. Lenstra, A. J., Buntjer, J. B., Janssen, W. F. On the origin of meat – DNA techniques for species identification in meat products. In: *Veterinary Sciences Tomorrow – Issue* 2. 2001. 15 p.
5. Pascoal, A., Prado, M., Castro, J., Cepeda, A., Barrosvelásquez, J. Survey of authenticity of meat species in food products subjected to different technological processes, by means of PCR-RFLP analysis. In *Eur. Food Res. Technol* 218, 2004. pp. 306 – 312.
6. Miller, S. A., Dykes, D. D., Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. In *Nucleic Acids Research*, 16 N3, 1987. pp. 1215.
7. Kocher,T. D., Thomas, K.W., Meyer, A., Edwards, V. S., Pääbo, S., Villablanca, X. F., Wilson, C. A. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences of the USA, 1989, 86(16),6196–62 00.
8. Gábor, M., Trakovická, A., Miluchová, M. Využitie variability cytochrómu b pri identifikácií živočíšnych druhov pomocou metód PCR-RFLP. In *Acta fytotechnica et zootechnica – special issue*, vol. 12, 2009, pp. 185 – 190.
9. Vincze,T., Posfai, J., Roberts, R.J. NEBcutter: a program to cleave DNA with restriction enzymes Nucleic Acids Res. 2003. 31: 3688-3691
10. Zehner, R., Zimmermann, S., Mebs, D., RFLP and sequence analysis of the cytochrome b gene of selected animals and man: methodology and forensic application, Int J Legal Med, 1998, 111: pp. 323–327