

Forensic identification of 12 mammal species based on size variation of mitochondrial cytochrome b gene using multiplex PCR assay

Črček M.^a and Drobnič K.^{b,c}

^a Student of Faculty of Chemistry and Chemical Technology, University of Ljubljana

^b National Forensic Laboratory, Police, Ministry of the Interior, 1000 Ljubljana, Slovenia

^c Faculty of Criminal Justice and Security, University of Maribor, Slovenia

mail: katja.drobnic@policijsi.si

In this study, we have applied the endpoint PCR method based on size variation of mitochondrial cytochrome b gene among mammals for identification of 17 mammals. We successfully identify 12 out of 13 mammal species common in the geographic region of Slovenia (Central Europe and West Balkan) using multiplex PCR with our own designed primers for 7 species (cat, pig, lynx, roe and red deer, wolf, bear) and a conventional system i.e. non-fluorescent primers and agarose gel electrophoresis. Only primer designed for identification of Eurasian lynx turned out to be unspecific. Probably, because it was designed on to sequence data from species close to Eurasian lynx while sequence data for Eurasian lynx was not available in databases at the time of our research. With this method we could not distinguish between subspecies (wolf from dog, and pig from wild boar). The species-specificity of PCR amplicons was further demonstrated by the ability of the assay to accurately identify species-specific DNA from mixed samples.



Introduction

Species identification of biological samples is one of the most important aspects in forensic science not only in routine casework analysis with human origin, but also in particular cases that include non-human remains. The specificity and efficiency of investigations are important for further legal proceedings. Conventional methods for species identification like immunological tests, morphological tests and preliminary enzymatic color tests are less and less used, because of their unspecificity and unreliability. The species identification must usually be accomplished through DNA analysis, mostly by DNA sequencing predominantly part of mitochondrial DNA (mtDNA) (D-loop, cytochrome b, cytochrome oxidase I and 12/16S RNA genes) using universal primers. Moreover, sequencing is a time consuming method, its limitation is with the interpretation of mixtures. Recently, a new approach based on the genetic differences among species using endpoint PCR method [1] or a realtime PCR [2] assay were established for the species identification. This approach is directly depending on primer design. The problem of cross-reaction, especially between species more closely related (e.g. roe and red deer), should be avoided. In this study we have applied the multiplex endpoint PCR method based on size variation of cytochrome b to the identification of 17 mammals belonging to 13 species common in the geographic region of Slovenia (Central Europe and West Balkan) using our own primer designed for 7 species. The multiplex assay was able to identify 12 out of 13 mammal species from one source sample.

Materials and methods

2.1. DNA sources. Biological samples were obtained from 17 mammals (dog, rabbit, cow, calf, horse, cat, fox, goat, sheep, wild boar, pig, roe and red deer, lynx, wolf, bear, fox) interesting for forensic investigation. The selected animals taxonomically belong to 13 species and two subspecies (pig and wild boar, dog and wolf). Blood samples of domestic animals were obtained from veterinarians and blood or tissue samples of wild animals from the Department of ecology and environmental protection, Faculty of biotechnology, University in Ljubljana. Using QIAamp DNA minikit (Qiagen, UK) and organic extraction method, a sufficient amount of DNA was extracted and then quantified with the spectrophotometer NanoDrop 3300 (Thermo Fisher Scientific Inc, USA).

2.2. Primer design. The partial cytochrome b sequences were obtained from the National Center for Biotechnology Information (NCBI) and aligned using Clustal W. We used non-fluorescent universal forward primer and species-specific reverse primers within cytochrome b (Applied Biosystems, CA). The universal primer was taken from Pääbo et al. [3]), seven species-specific primers (dog, rabbit, cow, horse, fox, goat, sheep) are from a previous study published by Tobe et al. [2] as well as primer specific for human [2]. Seven species-specific primers for cat, pig and wild animals (lynx, roe and red deer, wolf, bear) were self designed. Their map positions are between 471 and 717 bp on cytochrome b. The differences in the sizes of the PCR amplicons range from about 87 to 338 bp.

2.3. PCR amplification. Final PCR volume was 25 µl containing Taq Gold buffer 10x, 15mM MgCl₂, Taq Gold polymerase (2.5 U/µl) (Applied Biosystems, CA), dNTP (200µM), forward primer (0.8µM), one reverse primer (0.5 µM for roe and red deer; 0.25 µM for dog, goat, sheep, pig, wolf, bear, fox, horse, cat; 0.17 µM for cow and rabbit, 0.05 µM for human) for singleplex PCR and mix of reverse primers for multiplex PCR, and 1ng of sample DNA. Amplifications were proceeded after an initial denaturation step of 10 min at 95°C, for 35 cycles of 30s at 95°C, 30s at 60°C and 45s at 72°C, followed by a final elongation step of 20 min at 72°C. Amplification products were separated and visualized by electrophoresis of 4 µl aliquots on a 2% agarose gel. Their size was estimated by comparison with a GeneRuler 100 bp DNA ladder (Fermentas).

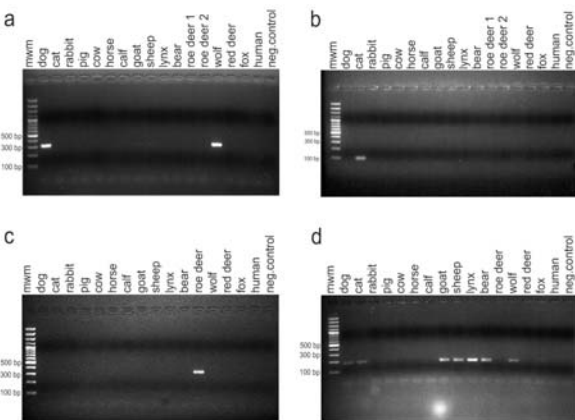


Figure 1: Amplification of cytochrome b for species identification. Single DNA of each animal species was subjected to singleplex PCR using universal and species specific primer for dog (a), cat (b), roe deer (c) and lynx (d).

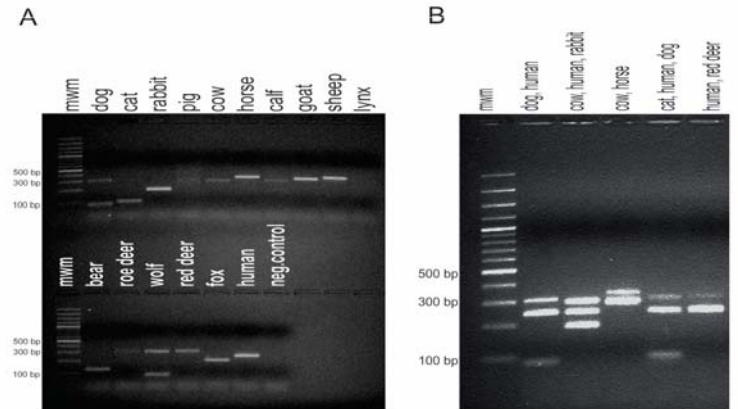


Figure 2: Multiplex amplification of cytochrome b for species identification. Single DNA of each animal species (A) and mixed DNA (B) were subjected to multiplex PCR using universal and all species specific primers (except primer specific for lynx).

Results and discussion

To search for variable regions, we compared the nucleotide sequences of cytochrome b in 7 mammals (cat, pig, lynx, roe and red deer, wolf, bear) that were available from the NCBI database. We found that the region between 471 and 717 bp is the most variable on cytochrome b [4] and useful for designating the species-specific primers. To check the specificity of species-specific primers and to confirm that the correct genomic sequences were being amplified in the multiplex PCR reactions, single PCR reactions were performed. The differences in the sizes of the PCR ranging from 87 bp to 338 bp permitted us to distinguish different sizes of PCR amplicons on agarose electrophoresis. Primers amplified the expected single PCR product successfully in all 17 mammal species. Species specificity was investigated using the same species-specific primer for amplification of DNA from different species. Twelve mammal species out of 13 were correctly identified (Figure 1a-d). Species identification was successful even between phylogenetically very similar species, i.e. roe and red deer (Figure 1c and 2A). Cross-reactivity with other species was detected with primers for lynx, wolf, dog and pig. Identification of lynx failed because the nucleotide sequences of lynx primer sequence differed by 5 nucleotides from those of other animals (dog, cat, goat, sheep, bear and wolf) (Figure 1d). Although we could not distinguish between subspecies wolf and dog (two fragment sizes of 90 bp and 306 bp, Figure 1a and 2A) and pig and wild boar (data not shown) using the species-specific wolf, dog and pig, the results are in concordance with our expectations. This method is not able to distinguish between subspecies. The same results were obtained using multiplex PCR (all primers except for lynx are included in amplification) (Figure 2A).

The applicability of the multiplex PCR assay for forensic purposes was demonstrated by simulated casework conditions where different sample mixtures (up to five DNA from different species) were investigated to identify the species. All product sizes of amplified regions in mixture of two/three mammals were as predicted (Figure 2B). The multiplex with five animals DNA added also provided the expected results, but with agarose gel electrophoresis we could not separate products that differ by a few nucleotides (data not shown). In further studies, we will try to achieve species identification from mixtures with multiplex PCR and capillary electrophoresis using fluorescent primers. This will provide more reliable interpretations of the results and it will allow us to identify a greater number of animal species with multiplex PCR.



References

- [1] S.S. Tobe, A. Linares, A multiplex assay to identify 18 European mammal species from mixtures using the mitochondrial cytochrome b gene, *Electrophoresis* 29 (2008) 340-347.
- [2] S. Kanthaswamy A. Premasathan, J. Ng J. Satkoski, V. Goyal, Quantitative real-time PCR (qPCR) assay for human-dog-cat species identification and nuclear DNA quantification, *Forensic Science International: Genetics*, in press.
- [3] S. Pääbo, J.A. Gifford, A.C. Wilson, Mitochondrial DNA sequences from a 7000-year old brain, *Nucleic Acids Res.* 16 (1988) 9775-9787.
- [4] T.D. Kocher et al, Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers, *Proc. Natl. Acad. Sci.* 86 (1989) 6196-6200.