

Application of molecular methods in forensic analyses of wild animals

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In European countries, hunting of wildlife is regulated by various directives. However, illegal hunting activities are becoming more common. To deal with wildlife crime cases, it becomes necessary to select the best molecular markers for the analysis of animal sex, species, and relationships identification. This study investigated potential markers within chromosomal and mitochondrial DNA (mtDNA) for animal forensics. By applying markers targeting nuclear DNA and mitochondrial DNA (the cytochrome *b* gene, hypervariable displacement loop (D-Loop) region, SRY and AMEL genes), the suspected illegal hunting cases were solved by the identification of wild species and sex. Currently, molecular forensic techniques are necessary before conclusions of its validation in forensic investigations.

Keywords: DNA, molecular methods, species identification, sex identification, RFLP

INTRODUCTION

In European countries, there are a number of directives that regulate hunting management principles and gaming methods for rational resource use. However, the European Union does not have one common law for unified management of hunting across all countries of the EU. The management of wildlife around the world (including Lithuania) is a serious concern in stopping illegal smuggling, hunting, and poaching of wild animals. The Rules of Hunting in the Territory of the Republic of Lithuania establish the list of game species and seasons of hunting of these animals, hunting methods, and their application deadlines, prohibited and permitted gear, a list of hunted game, annual hunting limits in individual units of the hunting area and safe behaviour in hunting and other hunting require-

ments (Order of the Minister of the Environment of the Republic of Lithuania on the Approval of the Rules of Hunting in the Territory of the Republic of Lithuania, 27 June 2000, No. 258). Forensic DNA analysis of wildlife animals is the application of DNA forensic methods to proper identification of parts of wildlife animals and their products. To apply the law, it is necessary to properly identify exhibits of each crime to the level of species and sex (Mitra et al., 2018).

Today, molecular markers play an important role in forensic in estimating an animal's sex, species, and relationships (Bellis et al., 2003). In some cases, it is difficult to perform an examination with very little or poor-quality test material (e.g., blood in sand). In cases of illegal hunting, several types of molecular markers are used, and it is important to mention that none of the molecular markers can be regarded as optimal for all applications. mtDNA sequencing and markers targeting nuclear DNA

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are used for the identification of a biological species from an unknown material (review by Arif et al., 2011). The cytochrome *b* (*cyt b*) gene is one of the most used genetic loci, applied in both taxonomy and forensic science for the purpose of species identification. The gene is 1,140 base pairs long in most vertebrates and shows little intraspecies variation, but sufficient interspecies variation. The gene is too long for typical PCR-based methods and, as many samples are degraded, only a part of the locus is used. DNA sequences at the start of the gene have become the favoured section for phylogenetics and species identification. The DNA sequences from any unknown sample from this part of the *cyt b* gene can be determined and compared to those on a DNA database to determine the most likely species from which the unknown sample originates (Linacre, 2012).

The purpose of this study is to investigate potential markers within chromosomal and mitochondrial DNA (mtDNA) for wildlife forensics.

MATERIALS AND METHODS

DNA extraction

To prove illegal hunting of animals, a wide variety of material has been collected for this study: pieces of tissue, blood, blood stains from various surfaces, soil with blood residues, bristles, bones, and the like. Depending on the type of material collected for research, different methods and kits such as Genomic DNA Purification Kit (Thermo Scientific™), GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific™), QIAamp DNA Mini Kit (Qiagen), PrepFiler™ Forensic DNA Extraction Kit (Applied Biosystems™) for DNA extraction were used. The quantity of DNA was measured, the quality was assessed, and DNA was stored at -20°C until PCR analysis.

Species identification

To identify a mammal's species from different samples, PCR amplification was performed using primers L14735 (5'-AAAAACCACCGTTGT-TATTCAACTA-3') and H15149 (5'-AAACT-

GCAGCCCCTCAGAATGATATTTGTCCTCA-3') of a partial cytochrome *b* gene sequence (308 bp) (Kocher et al., 1989; Spielmann et al., 2018).

In order to identify an animal species and using a short part of the target gene sequence, it may be difficult to distinguish related species or subspecies. For example, species-specific primers Pig F (5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3') and Pig R (5'-ACCATTGACTGAATAGCACCT-3') (Montiel-Sosa et al., 2000) designed for pork D-loop mtDNA were developed. The amplified band of 531 bp indicates that there is pork in the sample and this band is absent in PCR-amplified products from bovine, ovine, chicken, and human samples. To distinguish wild boar meat from pork, the restriction fragment length polymorphism (RFLP) analysis was done. Pork and wild boar amplified DNA can be easily distinguished by digestion with the BseDI (*BsaI*) (Thermo Scientific™) restriction enzyme for 8 h at 55°C.

Sex determination

The first sex determination test was developed by Pomp et al. (1995). The primer pair SRYB F (5'-TGAACGCTTTCATTGTGTGGTC-3') and SRYB R (5'-GCCAGTAGTCTCTGT GCCTCCT-3') was applied to investigate samples of unknown sex. An amplified product of 163 bp is only expected in males that carry the Y chromosome.

For amplifying the amelogenin gene of the sheep and the red deer, the set of primers SE47 (5'-CAGCCAAACCTCCCTCTGC-3') and SE48 (5'-CCCGCTTGGTCTTGTCTGTTGC-3') was used earlier (Ennis, Gallagher, 1994; Pfeifer, Brenig, 2005). In this study, individuals of the roe deer, the red deer, the moose, the wild boar, and the pig were analysed. One band 244 bp was produced by samples from females, samples from males amplified three bands (one non-specific band was observed; specific bands should be 244 and 190 bp).

Detailed PCR protocols used in this study of different targets are presented in the Table.

PCR amplification products were separated by gel electrophoresis in 0.5 × TAE buffer

Table. Detailed PCR protocols

Target	Components of PCR	PCR amplification steps
cyt <i>b</i>	1X DreamTaq Green PCR MM	95°C – 15 min
	0.5 µM Forward primer	94°C – 40 s
	0.5 µM Reverse primer	35 X 50°C – 1 min 20 s
	~100 ng DNA	72°C – 1 min 20 s
		72°C – 10 min
D-loop	1X MyTaq™ Reaction Buffer	92°C – 3 min
	2U MyTaq™ DNA Polymerase	92°C – 20 s
	2.5 mM Forward primer	30 X 58°C – 20 s
	2.5 mM Reverse primer	72°C – 30 s
	~100 ng DNA	72°C – 3 min
SRY	1X DreamTaq Green PCR MM	95°C – 5 min
	1 µM Forward primer	95°C – 30 s
	1 µM Reverse primer	35 X 56°C – 30 s
	~100 ng DNA	72°C – 30 s
		72°C – 5 min
AMEL	1X DreamTaq Green PCR MM	95°C – 10 min
	1 µM Forward primer	94°C – 50 s
	1 µM Reverse primer	35 X 56°C – 50 s
	~100 ng DNA	72°C – 1 min
		72°C – 2 min

(Thermo Scientific™) using 1.5–2% agarose at 100 V for 60 min, stained with ethidium bromide and visualised under UV light. The amplicons of species identification reaction (*cyt b*) obtained from amplification were purified using GeneJET Gel Extraction Kit (Thermo Scientific™) following the manufacturer's instructions. Purified PCR products were sent to a sequencing service (Macrogen, Amsterdam, Netherlands). The obtained sequences of different species were compared with sequences available from the GenBank database (www.ncbi.nlm.nih.gov) using the BLASTn tool of the National Center for Biotechnology Information. The identification and phylogenetic analysis of a mammal's species were conducted using MEGA X software (Kumar et al., 2018).

RESULTS AND DISCUSSION

mtDNA

Mitochondrial DNA (mtDNA) is widely used in forensic analyses because the cell has a high number of mtDNA copies, which facilitate

analysis from samples with highly degraded DNA, poor-quality test material, or trace amounts of DNA (Ballard, 2016). mtDNA is a relatively small, easy to isolate DNA molecule, and due to the presence of conserved sites such as the 12S, 16S, and cytochrome *b* (*cyt b*) gene, these fragments are widely used for species identification (Singh et al., 2017). The mtDNA control region spans over 1122 bp, evolves faster than the rest of the mitochondrial genome, and the three hypervariable regions of the control region, HVI, HVII, and HVIII, are also used for forensic investigation (Nakamura et al., 2008). In this study, fragments of the *cyt b* gene from potentially unknown mammals were amplified and sequenced. Comparison of the obtained data with the sequences uploaded in the NCBI Nucleotide database revealed that the tested animals were a red deer, a roe deer, a moose, and a wild boar. Compared sequence similarity ranged from 99 to 100%. Parson et al. (2000) conducted a study using *cyt b* gene. All *cyt b* gene sequences of the tested mammalian

species and the amphibians were found in DNA databases. The high similarity between the laboratory results and the database entry (99 and 100%) were also observed. These authors concluded that single nucleotide substitutions between the query and the subject sequence could be attributed to individual variation (Parson et al., 2000).

Restriction Fragment Length Polymorphism (RFLP)

The amplified product of mtDNA D-loop (531 bp) of the wild boar and the pig using primer pair Pig F and Pig R was observed in all tested samples. Based on these obtained results alone, it was not possible to identify a specific species, so RFLP analysis could be used to avoid sequencing. In this study, *Bsa*JI (*Bse*DI) restriction enzyme (Fig. 1) was used for amplified mtDNA digestion.

Digestion of the 531 bp amplified DNA fragment are shown in Fig. 2 and produced the ap-

pearance of one intense band of approx. 420 bp and one weak band of approx. 110 bp in pork meat and two intense bands of approx. 230, 210 bp and one weak band of approx. 110 bp in wild boar. In future work, capillary electrophoresis rather than the agarose gel should be used due to its better resolution.

A comparison of our results with Montiel-Sosa et al. (2000) showed that the PCR amplification products also produced a single band with the molecular size of 531 bp. *Bsa*JI endonuclease produced a single band with the molecular size of 470 bp in pork meat and undigested single band of 531 bp in the wild boar. However, the enzyme also produced smallest bands in the study by Montiel-Sosa et al. (2000), but the authors were not able to detect them and these results cannot be compared with our own results. More mutation regions in animals from different countries and continents could be identified if more restriction endonucleases are tested.

KC608841.1_Sus_scrofa_haplotype_H133_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
KC608844.1_Sus_scrofa_haplotype_H136_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
KC608840.1_Sus_scrofa_haplotype_H132_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
KC608843.1_Sus_scrofa_haplotype_H135_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
KP089976.1_Sus_scrofa_isolate_061_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
KP089977.1_Sus_scrofa_isolate_09_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
KP089966.1_Sus_scrofa_isolate_058_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
KP089967.1_Sus_scrofa_isolate_031_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
KP089968.1_Sus_scrofa_isolate_02a_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
MM625826.1_Sus_scrofa_domesticus_isolate_636465_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
MM625823.1_Sus_scrofa_domesticus_isolate_545556_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
MM625810.1_Sus_scrofa_domesticus_isolate_161718_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
MM625828.1_Sus_scrofa_domesticus_isolate_697071_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
AB015091.2_Sus_scrofa_domesticus_mitochondrial_DNA_D-loop_region_isolate_Satsuma_29	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
D42183.1_Sus_scrofa_domesticus_mitochondrial_DNA_for_repeat_sequence	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
MM625816.1_Sus_scrofa_domesticus_isolate_333435_D-loop_partial_sequence_mitochondrial	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
AB015092.1_Sus_scrofa_domesticus_mitochondrial_DNA_D-loop_region_isolate_Okinawa_native_pig	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240
D42180.1_Sus_scrofa_domesticus_mitochondrial_DNA_for_repeat_sequence	GAAAC CAGCAACCCGCTTGGCAGGGATCCCTCTTCGCTCCGGGCCATAAACCGTGGG	240

Fig. 1. Comparison of fragments of pig (*Sus scrofa domesticus*) and wild boar (*Sus scrofa*) mtDNA D-loop sequences from GenBank database (<https://www.ncbi.nlm.nih.gov/nucleotide/>). The recognition sites of *Bsa*JI (*Bse*DI) restriction endonuclease in wild boar sequences are marked in yellow

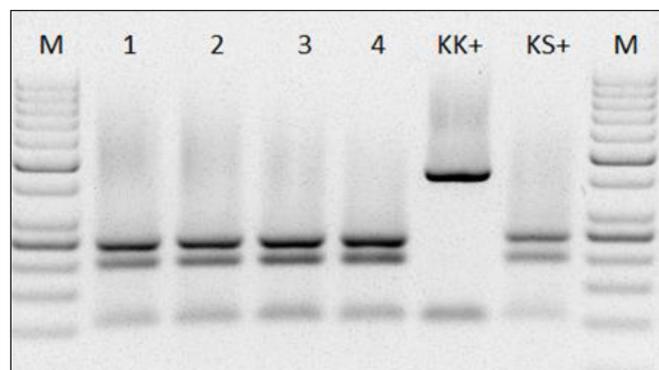


Fig. 2. Electrophoresis analysis in 2% agarose gels of the products obtained with *Bse*DI digestion of the amplified wild boar and pig mtDNA. Lines 1–4 represent samples from wild boar, line KK+ is a sample from pig, and line KS+ is a sample from wild boar. Lines M are 50 bp ladder DNA marker

Sex determination

Sex determination using molecular markers in domestic and wild animals are important in different fields: breeding (Sathasivam et al., 1995), meat production and processing (Zelenny et al., 2002), population genetics (Taberlet et al., 1993), forensics (Gupta et al., 2006), and others. Using several species of mammals, sex determination methods were developed by amplification of specific sequences of Y-chromosome or amplifying homologous fragments from both sex chromosomes (Fontanesi et al., 2008). The first molecular studies of sex determination were performed using the SRY gene in pigs (Pomp et al., 1995; Sathasivam et al., 1995). However, the disadvantage of this method is that the amplified product is only expected in males. Using this test alone can be problematic; it is not possible to distinguish the failure of PCR amplification from the correct identification of the samples as females (Fontanesi et al., 2008). For example, in this study, four animals (wild boar) for which the sex was not available and two animals for which the sex was available were analysed and compared (Fig. 3). In this study, these molecular markers for sex determination were also verified and validated for the roe deer, the red deer, and the moose (data not shown).

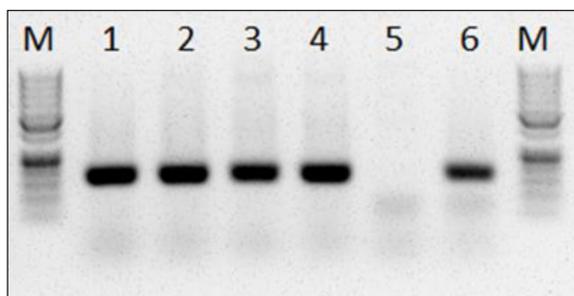


Fig. 3. Electrophoresis analysis in 1.5% agarose gel of sex determination based on the protocol described by Pomp et al. (1995). SRY gene PCR fragments specific to males (1, 2, 3, 4, 6) (163 bp) are shown. Line 1–4 samples come from wild boar of unknown sex, line 5 is a sample from a female wild boar, line 6 is a sample from a male wild boar. Lines M are 50 bp ladder DNA marker

Another gene used in sex determination cases in different mammalian species is amelogenin. X and Y chromosome-specific sequence differences of the amelogenin genes (AMELX and AMELY) were described (Fontanesi et al., 2008). The use of this gene in molecular analysis made the sex determination much less complicated, since only one pair of primers is required to amplify the different size fragments of the AMEL genes (Pfeiffer and Brenig, 2005). For example, four animals (roe deer individuals), for which the sex was not available, and five animals (roe deer and red deer individuals), for which the sex was available, were analysed and compared (Fig. 4). A single band for females and three bands (an additional non-specific band also was observed) for males were detected when analysing samples of different animals. Based on different banding patterns, there was no problem determining the sex of each individual.

The same results were observed in Pfeiffer and Brenig's (2005) study with sheep and red deer: a single band for females and two bands with one additional non-specific band for males were detected.

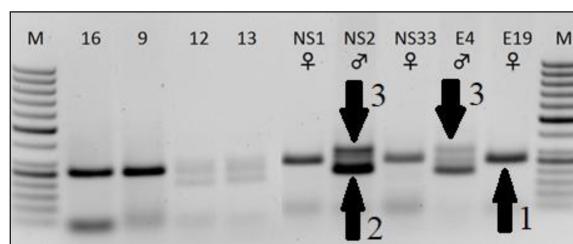


Fig. 4. Electrophoresis analysis in 1.5% agarose gel of sex determination based on the protocol described by Pfeiffer and Brenig, 2005. Arrow 1 indicates the AMELX band, arrow 2 indicates the AMELY band, and arrow 3 indicates non-specific bands in male roe deer and red deer. Lines 16, 9, 12, 13 are samples from red deer of unknown sex, lines NS1 and NS33 are samples from a female roe deer, line NS2 is a sample from a male roe deer, line E4 is a sample from a male red deer, and line E19 is a sample from a female red deer. Lines M are 50 bp ladder DNA marker

CONCLUSIONS

The molecular markers used in this study are suitable for species and sex identification. Even very small amounts of different testing materials can be used to identify specific animals (species and sex) hunted in illegal hunting.

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LAUKINIŲ GYVŪNŲ KRIMINALISTINĖ ANALIZĖ MOLEKULINIAIS TYRIMŲ METODAIS

Santrauka

Europos šalyse galioja įvairios laukinių gyvūnų medžioklės direktyvos. Visame pasaulyje (ir Lietuvoje) laukinių gyvūnų populiacijų valdymas kelia rimtą susirūpinimą – siekiama sustabdyti nelegalią laukinių gyvūnų kontrabandą ir medžioklę. Tiriant nusikaltimų laukinei gamtai bylas, būtina parinkti geriausius molekulinis žymenis gyvūnų lyties, rūšies ir giminystės identifikavimo analizei. Šiame tyrime buvo išanalizuoti chromosominiai ir mitochondrijų DNR (mtDNR) žymenys, kurie galėtų būti taikomi laukinių gyvūnų teismo ekspertizėje. Naudojant šiuos žymenis (mtDNR citochromo b geną ir variabilų D kilpos (D-Loop) regioną; SRY ir AMEL genus), buvo nagrinėjami įtariamos nelegalios medžioklės atvejai, identifikuotos laukinių gyvūnų rūšys ir nustatytos lytys. Molekuliniai DNR metodai yra būtini ir naudingi atliekant teisinę ekspertizę.

Raktažodžiai: DNR, molekuliniai metodai, rūšių identifikavimas, lyties identifikavimas, RFLP