Optimization of polymerase chain reaction for the identification of Roe deer, Saiga, and Siberian stag living in Kazakhstan

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Materials and Methods: Genomic DNA was extracted from meat and blood samples of animals killed by poachers using commercial kits. Three pairs of primers were designed and used to amplify the cytochrome b gene fragment of Roe deer, Saiga antelope, and Siberian stag.

Results: The proposed protocol allows amplification of specific PCR products of 542 bp with Roe deer DNA, 587 bp with Saiga DNA, and 525 bp with Siberian stag DNA. Specificity analysis showed no cross activity with DNA from other animal species. The detection limit of PCR ranged from 15.6 pg to 1.9 pg of DNA in 25 μL of the reaction mixture.

Conclusion: Sequencing the amplified products and subsequent comparison with the corresponding reference sequence showed a similarity ranging from 99.99% to 100%. The PCR based on the developed primers demonstrated high sensitivity and specificity when using DNA from homogeneous and heterogeneous animals.

Keywords: Argali, cytochrome b, identification, Roe deer, Saiga, Siberian stag.

Abstract

Background and Aim: One of the reasons for the decline in the number of wild species of artiodactyls is poaching and the illegal trading of animal products. Molecular genetic identification of animals from a biological sample effectively proves poaching cases and illegal trade of animal products. This study aimed to develop a polymerase chain reaction (PCR) test that allows for species identification of artiodactyl animals that are most often subject to poaching.

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search algorithm [12, 13]. The SNP has become one of the most reliable markers for identifying animals from a biological sample since these mutations are numerous and widespread in the genomes of most animal species. These markers are not only reliable and sensitive but also relatively inexpensive. Using the SNP fragment of the D-loop gene of mitochondrial DNA effectively identified 10 wild African animal species that were affected by illegal hunting [14]. The cytochrome b (Cyt b) gene is widely used to identify vertebrate species. The multiplicity nature of the mitochondrial genome, the high interspecies variability of the Cyt b gene, and the use of a large database of nucleotide sequences in phylogenetic relations, as a result, made it possible to use this marker in the development of PCR tests for species identification of animals [8, 15, 16]. The presence of conservative regions of the genome allows using universal primers for identifying an animal species by the cytochrome gene. Ideal for identifying an animal is primers that amplify regions of a gene that is sufficiently different between animal species. Equally important is the availability of the size of the amplified gene fragment for sequencing reaction [12]. Several primers have been developed to identify animal species based on the first 400 bp of the cytochrome gene [17].

Kazakhstan is implementing several programs to preserve the biological diversity of animal and plant fauna: Reserves are being created and legislation is being tightened for shooting and harming wildlife. Despite this, poaching cases are registered annually, and unfortunately, many of them remain unproven due to the limited evidence base. This is because species identification of animals is still carried out using immunological methods or based on morphological differences, which does not allow us to establish the species identity as a pure one, especially if poachers get rid of hooves, skins, heads, etc. Consequently, this study aimed to develop a PCR test that allows for species identification of artiodactyl animals that are most often subject to poaching.

**Materials and Methods**

**Ethical approval**

All of the samples were obtained from wild animals killed by poachers. All samples from domestic animals were obtained during the necessary diagnostic procedures by veterinary staff; therefore, no ethics committee approval was necessary.

**Study period, location, animal, and sample collection**

The study was conducted from January 2021 to December 2021, with target samples from the northern part of Kazakhstan. For DNA extraction, tissues of Roe deer (*Capreolus capreolus*), Saiga antelope (*Saiga tatarica*), Siberian stag (*Cervus elaphus sibiricus*), argali (*Ovis ammon*), sheep (*Ovis aries*), and cow (*Bos taurus*) were used. The species of artiodactyl animals of Kazakhstan used in this study, as well as the type and number of samples analyzed, are listed in Table-1.

Genomic DNA was extracted from meat samples of animals killed by poachers using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA). DNA extraction from blood was performed using QIAamp DNA Blood Kits (Qiagen, Germany), according to the manufacturer’s instructions.

**Primer design**

The search for sequences homologous to target genes containing consensus motifs was performed using the National Center for Biotechnology Information (NCBI) web resource (https://www.ncbi.nlm.nih.gov) and the PrimerSelect program (DNASTAR). The specificity of the primers, as well as the melting point, was tested in silico using the web service Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The designed primers are shown in Table-2.

**Polymerase chain reaction amplification**

The PCR amplification was performed in a volume of 25 µL containing 1 µL of genomic DNA at a concentration of 0.1 µM, 1 µL of forward primer at a concentration of 0.1 µM, 1 µL of reverse primer at a concentration of 0.1 µM, 2.5 µL of dNTP Mix (2 mM each) (Thermo Fisher Scientific, USA), 2.5 µL 10× Tag Buffer (Thermo Fisher Scientific), 1.5–2.5 µL 25 mM magnesium chloride (MgCl2) (Thermo Fisher Scientific), 0.5 µL Tag DNA polymerase (Thermo Fisher Scientific), and mQ up to 25 µL. Amplification was carried out in a T100 thermal cycler (Bio-Rad, USA) under the following conditions: Initial denaturation at 95°C for 5 min, then 30 denaturation cycles at 95°C for 1 min, annealing at 62–70°C for 1 min for Roe deer and 52–60°C for Siberian stag and Saiga, elongation at 72°C for 1 min, and final elongation at 72°C for 10 min. The PCR amplification products were purified using the QIAquick PCR Purification Kit (Qiagen), according to the manufacturer’s instructions.

**Sequencing of PCR products**

Sequencing of PCR products was performed using BigDye Terminator v3.1 kit (Applied Biosystems, USA), with primers used for PCR amplification according to the manufacturer’s instructions. The contig was assembled in LaserGene software (https://www.dnastar.com/software/lasergene/). Identification was performed using the NCBI web resource (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Results**

As a result of primer design, three specific pairs were selected, as shown in Table-2. DNA sizes
amplified by primers for Roe deer, Saiga, and the Siberian stag were 542, 587, and 525 bp, respectively.

The polymerase reaction was optimized to increase the specificity of amplification. The most effective parameters for amplifying the Cyt b fragment of Roe deer were a 1.5 mM MgCl₂ concentration in the reaction mixture and an annealing temperature of 61°C. The optimal parameters for Siberian stag and Saiga were 2.5 mM concentration of MgCl₂ in the reaction mixture and an annealing temperature of 55°C. The PCR with DNA from other animal species did not show product formation (Figure-1). The length of the amplified products corresponds to the predicted size. Sequencing the amplified products and subsequent comparison with the corresponding reference sequence showed similarities ranging from 99.99% to 100%. When determining the specificity of PCR, primers for one animal species were designed based on the material from other species of artiodactyl animals.

The sensitivity of the quantitative PCR reaction was tested using serial dilutions of DNA isolated from biological materials of artiodactyl animals. The PCR detection limit ranged from 15.6 pg to 1.9 pg of DNA in 25 µL of the reaction mixture (Figure-2).

### Discussion

The protection of wild artiodactyl animals, which are an important part of biodiversity, is a priority task for the conservation of genetic resources of rare and endangered species of the fauna in many countries of the world. To regulate human activities in the field of trade in products of animal origin, the veterinary, sanitary, and food legislation of the European Union includes several thousand regulations governing the veterinary and sanitary requirements of trade in animals and food of animal origin [19]. Despite the existence of legislative acts and various agreements on the trade of animals, poaching continues to cause significant damage to the population of wild artiodactyls. The main reason for the low efficiency of law enforcement is the lack of reliable methods for identifying animal remains by morphological features. Unlike morphological and spectrometric methods, modern DNA test systems make it possible to identify an animal species reliably [20]. Methods based on molecular markers are most widely used for identifying food and processed animal products. The advantage of molecular markers is associated with the need for small amounts

| Class | Order | Family | Species | Common name | Specimen type | Number of specimens |
|-------|-------|--------|---------|-------------|---------------|---------------------|
| Mammals | Artiodactyls | Deer | Capreolus pygargus | Roe deer | Meat | 6 |
| | | | Cervus elaphus sibiricus | Siberian stag | Blood | 1 |
| | Bovid | Saiga tatarica | Saiga | Bone | 1 |
| | | Ovis aries | Argali | Meat | 1 |
| | | Ovis aries | Sheep | Meat | 3 |
| | | Bos taurus | Cattle | Meat | 3 |

### Table-2: Primers for species identification of Saiga, Roe deer, and Siberian stag.

| Species | Common name | Accession number | Primers | Amplicon size |
|---------|-------------|------------------|---------|---------------|
| Capreolus pygargus | Roe deer | KT964433.1 | for 5’CATGGTGAAACTTTGGCTCTC 3’ rev 5’TGTTGGGTTGTTTGATCCTGTTTC 3’ | 542 |
| Saiga tatarica | Saiga | JX177502.1 | for 5’GACACAGCAGACAGCATCCTCTC 3’ rev 5’GGGTCCTCAAGCAGGCTCT 3’ | 587 |
| Cervus elaphus | Siberian stag | NC_007704.2 | for 5’CGGCGCATCAATATTTTTTCTCTG 3’ rev 5’TTGCTGGGGTGTAGTTATCTGGA 3’ | 525 |

**Figure-1:** DNA amplification of Roe deer, Saiga, Siberian stag, argali, sheep, and cow by polymerase chain reaction based on the developed primers. Lanes 1, 9, and 16: Roe deer; lanes 2, 8, and 17: Saiga; lanes 3, 10, and 15: Deer; lanes 4, 11, and 18: Argali; lanes 5, 12, and 19: Sheep; lanes 6, 13, and 20: Cow; and lanes 7 and 14: Molecular marker.
of DNA, the ability to identify an animal by several markers, and the use of a PCR method that allows obtaining both qualitative and quantitative results. The most popular markers for tracking and controlling products of animal origin, such as SSR and SNP, are highly informative and can identify animals between species and within species [21].

To identify animal species, a standardized Cyt b gene has been proposed. This marker proved to be effective in identifying animals in epidemic conditions and illegal trade in wild animals. The mammalian Cyt b gene is the least susceptible to mutations that change the amino acid sequence of a protein, rendering it suitable for identifying unique genetic sequences. Multiplex PCR analysis based on four pairs of primers for the gene of buffalo, cattle, pig, and duck made it possible to determine falsification in all types of food products with high accuracy [22]. Using the Cyt b gene and direct PCR, a rare paka animal was identified in the illegal meat trade. The method based on a single-nucleotide substitution of the Cyt b gene proved effective and differentiated paka meat with 100% certainty [14]. The presented results demonstrate the usefulness of PCR analysis based on primers for the Cyt b gene in the authentication of meat and meat products in the laboratory. Applying modern developments in the field of high-speed polymerases render identifying an animal species in the field a real possibility [23].

To identify Roe deer, Saiga, and Siberian stag living in Kazakhstan based on biological samples, three pairs of primers were developed to amplify fragments of the Cyt b gene ranging in size from 525 to 587 bp. Analysis of amplified and reference sequences showed high genetic similarity of the amplified and reference sequences indicate the specificity of the designed primers. This PCR method, which was optimized to determine the Cyt b gene of Roe deer, Saiga, and Siberian stag in biological samples in the laboratory, can also be used to detect Roe deer, Saiga, and Siberian stag meat in food adulteration.

**Conclusion**

Preservation of the number of wild artiodactyl animals is an urgent task for Kazakhstan. To identify Roe deer, Saiga, and Siberian stag from biological samples, three pairs of primers were developed to amplify fragments of the Cyt b gene ranging in size from 525 to 587 bp. Analysis of amplified and reference sequences showed high genetic similarity of the amplified and reference sequences indicate the specificity of the designed primers. This PCR method, which was optimized to determine the Cyt b gene of Roe deer, Saiga, and Siberian stag in biological samples in the laboratory, can also be used to detect Roe deer, Saiga, and Siberian stag meat in food adulteration.

**Authors’ Contributions**

KM and YR: Contributed to conceptualization and design of the study. DK, SZ, and KT: Sampling, DNA extraction, PCR optimization, and sequencing. AS, and KMukanov: Troubleshooting of PCR technique and helped in analysis of PCR results. KM and AS: Analyzed and interpreted the data. KM: Prepared and wrote the original draft. KMukanov and YR: Reviewed and edited the manuscript. All authors have read and approved the final manuscript.

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**Competing Interests**

The authors declare that they have no competing interests.
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