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RESEARCH ARTICLE

PCR-BASED IDENTIFICATION OF MEAT SPECIES USING CYTOCHROME B

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ABSTRACT

This study aimed to develop a PCR-based technique for the identification of meat and blood samples from major domestic animal species and wild boar using the mitochondrial Cytochrome b (Cyt b) gene. DNA was extracted from meat and blood samples following standard protocols and amplified through gradient PCR using species-specific Cyt b primers. The optimized gradient temperatures successfully amplified Cyt b across multiple species, including cattle/buffalo, goat/sheep, horse/donkey, chicken, and pig, though amplification patterns differentiated only at the genus level (e.g., horse/donkey, sheep/goat, and cattle/buffalo). The results demonstrated that gradient PCR can serve as a reliable approach for species differentiation and may be further utilized to develop multiplex PCR assays for simultaneous identification of multiple species in a single reaction. This technique provides practical utility for food authentication, wildlife monitoring, and forensic investigations.

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Introduction

Meat and meat by-products play a significant role in human nutrition as they provide high-quality proteins, essential amino acids, and important micronutrients (Balakrishna et al., 2019). Ensuring the authenticity of meat is therefore critical for consumers, regulatory agencies, and the food industry, particularly in cases of mislabeling, adulteration, and contamination (Girish et al., 2013). In recent years, the authentication of meat species has gained global attention due to increasing reports of economically motivated food fraud. According to recent global assessments, more than 400 food-fraud incidents were reported across 66 countries in 2023 alone, with meat and meat products accounting for approximately 15% of all documented adulteration cases. Long-term analyses of the international beef supply chain have also reported over 400 meat-fraud incidents in a 20-year period, while large regional studies have shown that meat, milk, and oils together constitute up to 80% of food-fraud violations. These observations highlight the persistent vulnerability of meat supply chains worldwide and the need for robust authentication methods.

Genetic markers have become the preferred tools for species identification due to their specificity and reliability. Among these, mitochondrial genes such as Cytochrome b (Cyt b) and Cytochrome c oxidase subunit I (COI) are the most widely used because of their conserved nature and high copy number in cells (Silva-Neto et al., 2016). Earlier methods for detecting meat adulteration relied on chemical, anatomical, or immunological assays; however, these approaches lack sensitivity and are often unsuitable for processed meats. DNA-based

methods—particularly polymerase chain reaction (PCR)—have emerged as the gold standard for species identification because of their robustness, thermostability of DNA, and ability to detect species in raw or cooked meat samples (Balakrishna et al., 2019).

Various PCR-based techniques have been explored for meat authentication, including loop-mediated isothermal amplification (LAMP) (Balakrishna et al., 2019), PCR-RAPD (Saez et al., 2004), real-time PCR (Orbayinah et al., 2019), multiplex PCR (Ali et al., 2015), and species-specific PCR (Kumari et al., 2019). The Cyt b gene, in particular, has been extensively targeted for identifying a wide range of species, including *Bos taurus* (Kumari et al., 2019), *Bubalus bubalis* (Hossain et al., 2016), and *Sus scrofa* (Hossain et al., 2016). Several studies have demonstrated the effectiveness of Cyt b primers for differentiating both domestic and non-domestic species. However, despite its utility, no comprehensive study has been conducted on Cyt b-based species identification in Pakistan.

The present study aimed to design and evaluate species-specific Cyt b primers for reliable identification of eight commonly consumed or relevant species in the Khyber Pakhtunkhwa province of Pakistan. These included *Capra aegagrus hircus* (goat), *Ovis aries* (sheep), *Bubalus bubalis* (buffalo), *Bos taurus* (cow), *Sus scrofa* (pig), *Equus caballus* (horse), *Equus asinus* (donkey), and *Gallus domesticus* (chicken). This work provides foundational data for developing robust PCR-based assays that can support meat authentication, food safety monitoring, and regulatory enforcement in the region.

Materials and Methods

Sampling

Eight different animal species were selected for this study. Meat samples of *Capra aegagrus hircus* (goat), *Ovis aries* (sheep), and *Gallus domesticus* (chicken) were collected from the meat market at Bacha Khan Chowk Peshawar (34.0170° N, 71.5699° E). Meat samples of *Bubalus bubalis* (buffalo) and *Bos taurus* (cow) were taken from the slaughterhouse at Charsadda Road, district Peshawar. Meat samples of *Sus scrofa* (pig) (wild boar) were collected at the Tarbella region of district Swabi (34° 5' 32" North, 72° 41' 7" East). Whereas blood samples of *Equus caballus* (horse) and *Equus asinus* (donkey) were collected from Animal Brooke Hospital, Landai Sarak, Charsadda Road, Peshawar. Meat samples from all species were collected in sterile sampling containers and transported under controlled conditions to the Genomic Laboratory, Veterinary Research Institute, Peshawar (34.0170° N, 71.5699° E), and stored in an ultralow temperature freezer (-80°C) till further process.

Chemicals and reagents

Chemicals and reagents used in the study were procured under ADP scheme No. 32/170091 "Characterization of Cattle Genetic Resources of Khyber Pakhtunkhwa using Genetic Markers and Molecular Techniques, 2017-2021 (Phase-I)". The chemicals used in the current study belonged to Solisbiodyne®, Macrogen®, Sigma Aldrich®, Invitrogen®, and Thermo Fischer Scientific®.

DNA extraction

The DNA isolation was carried out using Gene All® Exgene™ Tissue SV mini (104-101, 104-152) according to the manufacturer's instructions. The extracted DNA was quantified using nanodrop (TiterTek® Germany). The extracted and quantified DNA was confirmed on 2% agarose gel and stored in DNase/RNase-free 1.5 ml Eppendorf tubes at -80°C till further studies.

Primers design

The primers used in the current study were synthesized by Macrogen®. The primers used in the study are given in Table 1.

Table 1. List of primers used in the present study

Common forward sequence	Forward primers 5 - GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA-3	**Amplicon size (bp)**	**References**
Goat	5-CTCGA CAAATGTGAGTTACAGAGGGA-3	331 bp	(Matsunaga et al., 1999; Arif and Abbas, 2015; Di Pinto et al., 2015)
Chicken	5-AAGATACAGATGAAGAAGAATGAGGCG-3	227 bp	
Cattle	5-CTAGAAAAGTGTAAGACCCGTAATATAAG-3	274 bp	
Sheep primers	5-CTATG AATGCTGTGGCTATTGTCGCA-3	331 bp	
Pig primers	5 –GCTGATAGTAGATTTGTGATGACCGTA-3	398 bp	
Horse	5 -CTCAGATTCACTCGACG AGGGTAGTA-3	439 bp	

PCR condition

Purified DNA of various species was subjected to PCR reaction using the mentioned primers against each species. A total of 25µL reaction size was constituted in a PCR tube (10µL master mix (SSO fast®), 5µL template DNA, 1.5µL forward and reverse primers, and 7µL PCR water). The amplification was carried out in a Bio-Rad Thermal Cycler® with the following temperatures: 95°C (initial denaturation) for 3 minutes, 94°C for 1 minute (cyclic denaturation), Tm (specific to each primer), 72°C (cyclic extension) for 1 minute, and 72°C (final extension) for 7 minutes.

The PCR product was run on 2% agarose gel in the gel electrophoresis system (at 30 mA and 220 volts for 30 minutes, Major Science®) and was subsequently visualized by UV illumination. The picture was obtained in the Gel documentation system (Fas Gene FAs Digi Nippon®, Germany). All the steps involved in DNA extraction (from both the blood and meat), PCR reaction, and gel documentation were performed in the genomic laboratory, Veterinary Research Institute, Peshawar.

Ethical Approval

Ethical approval for the current study was given by the Veterinary Research Institute (VRI-09), dated 25-08-2017. All the procedures for handling of blood and meat samples and DNA extraction were performed as per SOPs of the Genomic Laboratory, VRI, Peshawar.

Results & Discussion

Primer Design and DNA Extraction

Meat identification through DNA procedure is an essential approach for observation and diagnosis in various animal species (Eaton et al., 2010). Especially, the PCR-based identification technique through the cytochrome (Cyt-b) gene in different species is an important tool to develop for the meat specification (Silva-Neto et al., 2016). For this study, the primer was designed to have its initial step for DNA analysis using the PCR method **(Table 1).** In the design primer for all the species, we used common forward primers; however, reverse primers for the species like *Capra aegagrus hircus* (goat), *Ovis aries* (sheep), *Gallus domesticus* (chicken), *Bubalus bubalis* (buffalo), *Bos taurus* (cow), *Sus scrofa* (pig) (wild boar), *Equus caballus* (horse), and *Equus asinus* (donkey) mitochondrial Cyt-b gene (Table 1). The selection of this gene was based on the degree of intraspecies variability in the Cyt-b (Andrejevic et al., 2019). Moreover, Cyt-b has shown a larger number of DNA copies for every cell; therefore, it can survive DNA copies of tissue during extreme conditions such as sterilization and boiling (Andrejevic et al., 2019). The current study demonstrates for the first time in Pakistan by conducting various species identifications through PCR. For this purpose, whole blood/meat samples obtained from various sources as mentioned were processed for DNA extraction using standard protocols. Meat samples were obtained from cattle, buffalo, sheep, goats, chickens, and pigs and were subjected to DNA extraction, whereas the meat of horses and donkeys was not available; hence, blood samples were collected from horses and donkeys and subjected to DNA extraction. About 200 mg-300 mg of meat tissue was triturated in a clean and sterile (autoclaved) pestle & mortar and was ground into a powder. The powder was collected in a 1.5 mL Eppendorf tube. About 200µL of TL buffer was added to the powder and vortexed for 15 seconds.

After vertexing, 20µL of proteinase K solution was added, mixed completely by vortexing, and then incubated at 56°C for 20-30 minutes for complete lysis. The sample was then subjected to the protocol as described in the user manual (Gene All® Exgene™ Tissue SV mini (104-101, 104-152)). The extracted DNA was quantified using Nanodrop (Titertek®).

PCR amplification for detection of species

In recent years the confirmation of species has been growing in importance in the world. According to recent studies, there are 1.8 billion Muslims living on the globe. Islam prohibited haram meat and meat by-products for food utilization. The Islamic act prevents the consumption of certain species of meat, such as pork, cat, dog, donkey, and certain species of birds (Rahman et al., 2014). However, in recent years, these animal meats are mixed with halal foods due to cheap availability in certain regions. Reports have been made for the consumption of horse meat (Premanandh, 2013), pigs and rats (Van der Spiegel et al., 2012) mixing with halal foods has put the Muslim community on high alert (Ali et al., 2015). Therefore, the confirmation of multiple species of meats for Muslim consumers' traceability in a robust and authentic method is an auspicious need.

Previous studies have highlighted the application of the Cyt b gene for diagnosis and as a molecular tool for the identification of various species, such as beef (Hossain et al., 2016; Kumari et al., 2019); buffalo (Hossain et al., 2016); pig (Hossain et al., 2016), cat, dog, monkey, and rat meat (Ali et al., 2015); sharks (Kumar et al., 2007); seals (Malik et al., 1997); marine turtles (Moore et al., 2003); snakes (Yau et al., 2002); manatees (Ferreira et al., 2011); sheep, etc. (Andrejevic et al., 2019). The DNA obtained from each species was subjected to amplification using a thermal cycler. The following results were obtained. Initially, amplification of the Cyt B gene was unsuccessful at the previously tested annealing temperatures. So, we opted for gradient PCR for the amplification of Cyt b in this study. The gradients at which the amplification was successful are as: for cattle (cow) gradient was set at 52ºC to 63°C **(Figure 1)**, for chicken gradient was set at 58ºC to 68ºC **(Figure 2),** for buffalo gradient was set at 50 ºC-62ºC **(Figure 4),** for sheep the gradient was set at 62ºC-68ºC **(Figure 3)** and for goat the gradient was set at 48ºC-64ºC **(Figure 4)**, for pig, the gradient was set at 50ºC-58ºC **(Figure 6),** whereas for Horse the gradient was set at 58ºC-66ºC and for Donkey the gradient was set at 50ºC-58ºC **(Figure 5)**.

The annealing temperatures at which the amplification of the Cyt b gene in different species were successful are given in table 2 and figure 7. In cattle (cow) Cyt b amplified at annealing temperatures 63ºC, 58.8ºC, and 55.7ºC **(Figure 1)**, In chicken Cyt b amplified at 61ºC, 62.9ºC, 65.6ºC and 68ºC **(Figure 2),** In sheep Cyt b amplified at 63.8ºC only **(Figure 3),** In goat Cyt b was amplified at six different temperatures, i.e., 48°C, 49°C, 51.8°C, 55.5°C, 60°C, and 63.9°C **(Figure 4).** In buffalo, Cyt b was amplified at 51.8°C, 55.5°C, and 60°C **(Figure 4).** In horses and donkeys, Cyt b amplified simultaneously at four different temperatures, i.e., 50°C, 51°C, 52.9°C, and 55.7°C **(Figure 5),** whereas in pigs, Cyt b amplified at two temperatures only, i.e., 61.5°C and 61.6°C **(Figure 6).** The amplified products obtained were subjected to electrophoresis on 2% agarose gel and were visualized under UV in the gel documentation system. Different band lengths for each species were detected. The band length obtained for sheep/goat was 331 bp, for horse/donkey it was 439 bp, for cattle/buffalo it was 274 bp, and for chicken it was 227 bp, whereas for pig, it was 398 bp **(Figures 1-7)**. The protocol described in the present study is a simple, reliable, and reproducible procedure for the identification of different species in Khyber Pakhtunkhwa (**Figure 7**).

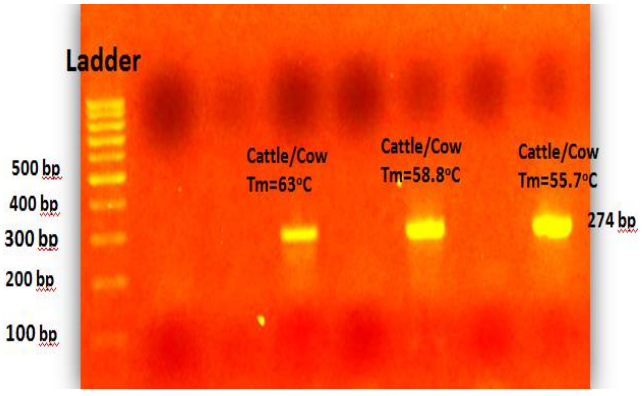


Figure 1. Gradient PCR for Cytochrome B Gene Amplification of cattle (cow) gene.

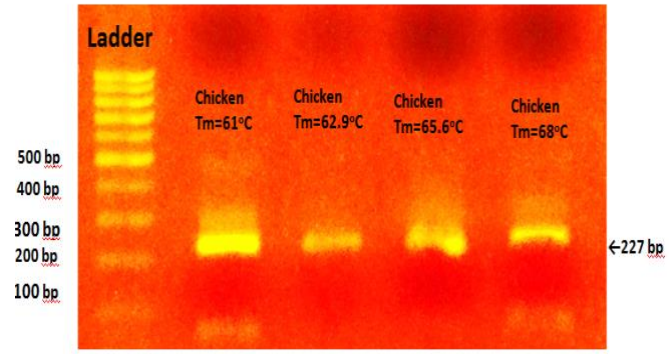


Figure 2. Gene amplification of Chicken (Broiler) gene.

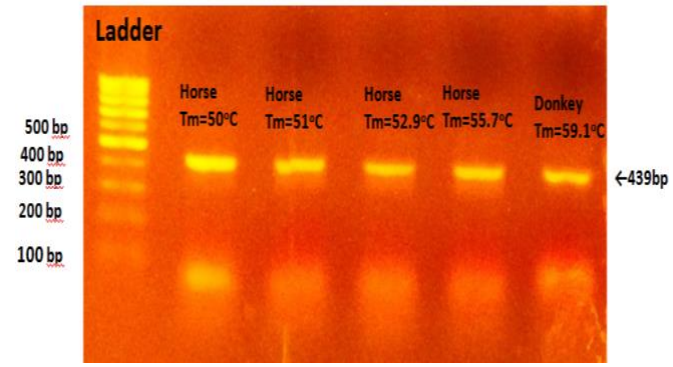


Figure 3. Gene amplification of Horse.

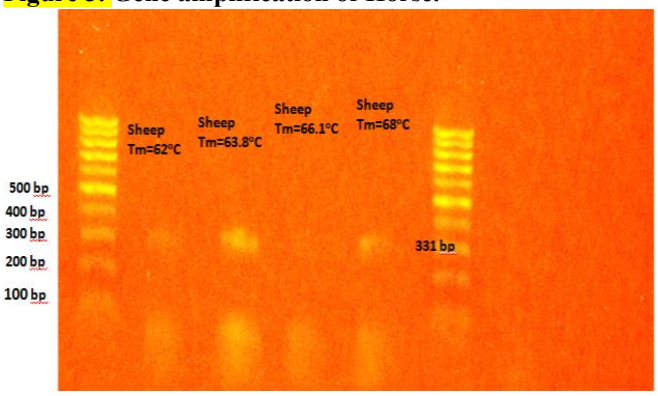


Figure 4. Gradient PCR for Cytochrome B amplification of sheep gene.

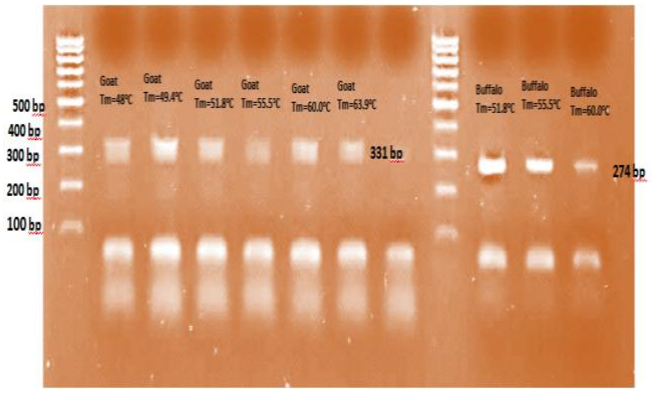


Figure 5. Gradient PCR for Cytochrome B amplification of goat (lanes 2-8) and buffalo (Lane 10-12).

Our result was similar to that of previous studies. Sing et al demonstrated that PCR assay for identification and differentiation of meat in Cattle, Sheep, Goat, Pig, and Chicken through Cyt b gene (Satish et al., 2011). Moreover, Kumar et al. (2014) also developed an experimental tool to identify common species beef, carabeef, chevon, mutton, and pork meat in a PCR assay through the Cyt b gene. Furthermore, Ali et al. (2015), also developed a tool for different species like Cat, Dog, Pig, Monkey and Rat meat through the Cyt b gene for identification of halal meat because these species are forbidden in Islam (Ali et al., 2015).

Conclusion

This pilot study demonstrates that gradient PCR targeting the Cytochrome b gene can reliably differentiate meat samples at the intra-genus level, particularly for cattle/buffalo, sheep/goat, and horse/donkey. The approach offers practical utility for halal authentication, prevention of meat adulteration, and routine food safety monitoring in local and regional supply chains. However, the method is limited in its ability to distinguish closely related species. For accurate intra-species identification, further investigation of the Cytochrome c oxidase subunit I (COI) gene—through extensive sequence alignment to identify conserved and variable regions—will be required to design highly specific primers for each species. Future studies should also evaluate the development of multiplex PCR assays to enable simultaneous detection of multiple species in a single reaction, improving cost-effectiveness and laboratory efficiency. Additionally, validation across processed meats and larger sample sizes will enhance the robustness and applicability of this technique for broader regulatory and forensic use.

Authors' contribution

MTZ designed the overall study. MTZ, MHR, designed and conducted the molecular study at the genomic laboratory, Veterinary Research Institute, Peshawar. OU, MTZ, SSAS, MTK analyzed the results. MTZ, OU wrote the manuscript. AU, OU, IA critically analyzed the manuscript and suggested a title for the manuscript. All the authors read and approved the manuscript for publication.

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Conflict of Interest

The authors of the manuscript declare that there is no conflict of interest.

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