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PD Bhole
Department of Veterinary Anatomy and Histology, Nagpur Veterinary College, Nagpur, Maharashtra, India

Rupali Charjan
Department of Veterinary Anatomy and Histology, Nagpur Veterinary College, Nagpur, Maharashtra, India

SB Banubakode
Department of Veterinary Anatomy and Histology, Nagpur Veterinary College, Nagpur, Maharashtra, India

NC Nandeshwar
Department of Veterinary Anatomy and Histology, Nagpur Veterinary College, Nagpur, Maharashtra, India

UP Mainde
Department of Veterinary Anatomy and Histology, Nagpur Veterinary College, Nagpur, Maharashtra, India

Anand Singh
Department of Veterinary Anatomy and Histology, Nagpur Veterinary College, Nagpur, Maharashtra, India

Ayesha Boddupalli
Department of Veterinary Anatomy and Histology, Nagpur Veterinary College, Nagpur, Maharashtra, India

Corresponding Author:
PD Bhole
Department of Veterinary Anatomy and Histology, Nagpur Veterinary College, Nagpur, Maharashtra, India

Species identification by PCR method using "Cytochrome B" As a molecular marker

PD Bhole, Rupali Charjan, SB Banubakode, NC Nandeshwar, UP Mainde, Anand Singh and Ayesha Boddupalli

Abstract
Identification of species of animals from a very small size of recovered biological sample from the site of crime has received great importance in vetero-legal cases to curb the practices of cruelty against animals and to lock up criminals. DNA is used to identify Buffalo, Cattle, Sheep, and Goat species. Six samples of each species were collected and DNA was extracted. The obtained DNA was then subjected to PCR, by using the species-specific Cytochrome b gene as a marker. The processed PCR outcome was electrophoresed in agarose gel and illuminated with a UV transilluminator. The illuminator revealed fragments at 106 bp, 163 bp, 232 bp, and 308 bp for Buffalo, Cow, Sheep, and Goat, respectively.

Keywords: Species identification, PCR, cytochrome b gene

Introduction
There are ever-growing numbers of cases of animal cruelty in which animals are illegally slaughtered for meat. Cow slaughter is one of the most important concern that can make a hawk from a religious point of view. Butchers perform practices such as mixing cheaper meat with costly meat in order to maximize profit. (Apparao *et al*., 1994; Panwar *et al*., 2015; Sangthong *et al*., 2021; Bhole *et al*., 2022) [1, 4, 6, 2]. This particular kind of fraud needs to be dealt legally since it is of utmost importance to public health, economy, and religion. After the crime has been committed various biological materials such as blood, skin, bone, muscle, organs etc. are left behind, which can prove to be conspicuous enough to imprison the criminal. The police department, NGO like Gorakshan, etc. send such recovered samples to the Department of Veterinary Anatomy for species identification.
When such biological materials are brought to the laboratory for analysis, it is likely to undergo putrefaction and makes it difficult for species identification based on the gross anatomical structure. Moreover, it is not possible to identify the species on gross anatomical features from a very small pieces of meat, bone, and other organs. Moreover, the Vetro-legal report on the species identification of the animal based on gross anatomical structure cannot be considered fully authentic and precise. (Rupali *et al*. 2022) [5]. Such reports aren't considered in a court of law, hence criminals cannot be imprisoned.
To tackle this issue, it is necessary to develop biological markers for species identification using molecular biology techniques. Molecular genetics has a lot of potential in terms of biological structure conservation. One such use is the development of species-specific genetic markers. These markers can be utilised to determine the species, ensuring absolute authenticity. Hence the present work is being undertaken to isolate DNA from blood for identification of species of animal by using conventional PCR assay.

Materials and Methods
For the present work, blood samples of six individual animals of each species i.e. cattle, buffalo, sheep, and goat were collected in 2 ml of K3EDTA tubes. These collected samples were kept in a freezer at 4 °C. High-quality genomic DNA was extracted by using DNeasy Blood and Tissue Kit (Qiagen, Germany) as per the standard protocol provided by the company.

The genomic DNA isolated from the blood samples was checked for its concentration. The extracted DNA was then added to a Nanodrop Spectrophotometer (Eppendorf Biospectrometer). 2 µl of extracted DNA was loaded in the cuvette. The cuvette was then placed in the cuvette shaft and was covered by a cuvette shaft cover. To check the purity of DNA the OD ratio (260/280) was within 1.7 to 1.9. The concentration of the extracted DNA was more than 50 ng/µl. DNA of good purity and concentration was used for further study**.**

The published primer pairs (Sangthong *et al.* 2021) [6] based on the species-specific Cytochrome b gene sequences of Buffalo, Cattle, Goat, and Sheep were used for species identification in the present study. The following primers were used during the present study:

Buffalo-specific forward primer: CATACATCCAAACAACGAAGTATG
Cattle-specific forward primer: TAGGAGGAGTACTAGCCCTAGCCT
Goat-specific forward primer: CCTCACATTAAACCTGAGTGGTAT
Sheep-specific forward primer: CATGCTACTAGTACTATTCACGCCT
Common reverse primer: CTGGYTGKCCTCCAATYCATG

The quantified DNA was subjected to PCR in a 25µl reaction mixture in a PCR tube, which was then placed in the PCR machine at the cyclic condition as mentioned below;

Table 1: PCR machine at the cyclic condition as mentioned below

Stages		Temperature (°C)	Time
Stage 1	Initial denaturation	94 °C	2 min
Stage 2 (35 cycle)	Denaturation	94 °C	30 sec
	Annealing	61 °C	30 sec
	Extension	72 °C	30 sec
Stage 3	Final Extension	72 °C	30 sec

Agarose gel electrophoresis

Two per cent agarose gel was prepared by adding ethidium bromide to agarose and TAE buffer. The agarose gel was left to set and then it was loaded in submersible electrophoresis. The gel was added with 1 X Tris Acetate EDTA at a height of roughly 1 mm. The wells of the gel were filled with the PCR amplicon and ladder. Finally, the electrophoresis was conducted for 18 minutes, at 110V to get the band pattern.

UV transilluminator:

The agarose gel was carefully lifted and covered with due care, to observe under UV transilluminator by wearing safety goggles.

Results and Discussion

During the current study, the Cytochrome b gene exhibited fragments in UV transilluminator at 106 bp, 163 bp, 232 bp, and 308 bp for Buffalo, Cattle, Goat, and Sheep, respectively. The findings of the present study regarding the appearance of bands were similar with those reported by Sangthong *et al.* (2021) [6]. On the other hand, despite using the Cytochrome b gene as a primer, Jain et al. (2007) [3] noticed a band at 274 bp for Cattle and Buffalo which is not in agreement with the findings of the present study. This variation in band patterns of Cattle and Buffalo reported by Jain *et al.* (2007) [3] could be attributed to variations in the primer sequence. The findings of the current study are also in contradiction with those reported by Panwar *et al.* (2015) [4], who found the band at 157 bp for Goat and 331 bp for Sheep. This variation in the outcome could also be attributed to variations in the primer sequence. Hence, it can be concluded that even if the targeted gene is the same, there can be variations in the appearance of the band patterns, if the sequence of primer is different.

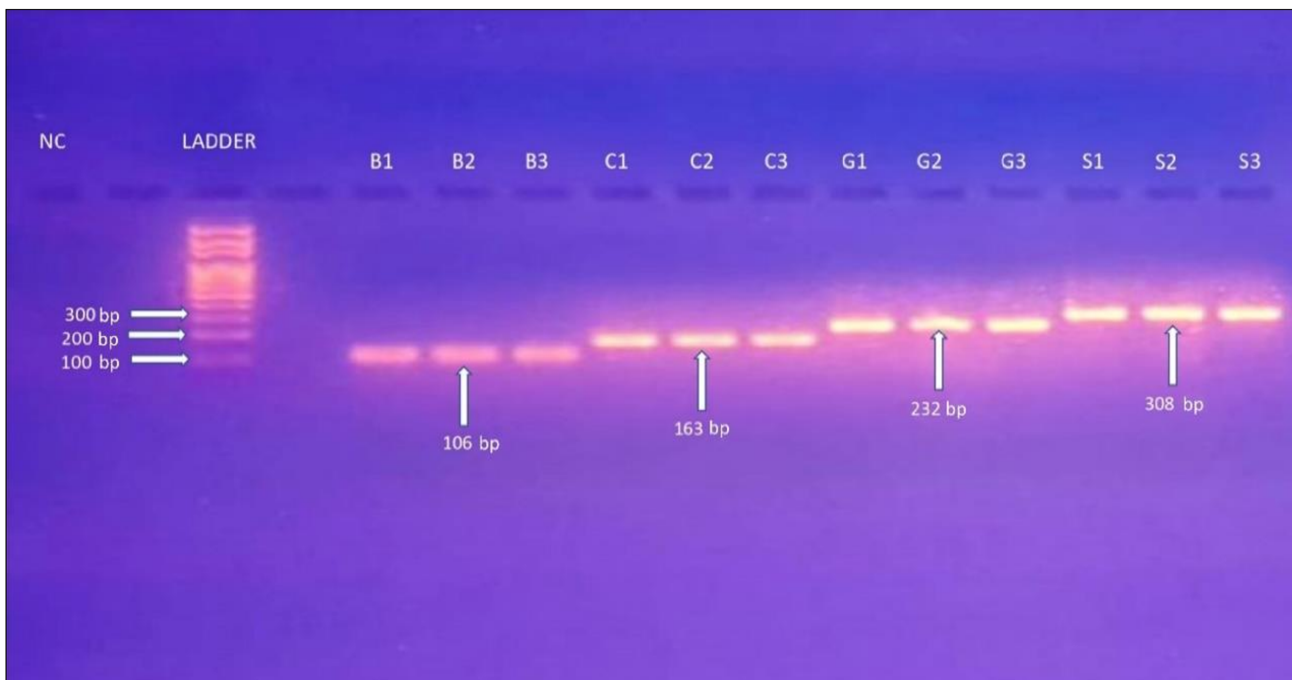

Fig 1: Amplification of species-specific Cytochrome b gene of all the species under study from the blood sample.

NC = Negative control
B1 = Buffalo blood 1
B2 = Buffalo blood 2
B3 = Buffalo blood 3
C1 = Cattle blood 1
C2 = Cattle blood 2
C3 = Cattle blood 3

Ladder = 100 bp ladder
G1 = Goat blood 1
G2 = Goat blood 2
G3 = Goat blood 3
S1 = Sheep blood 1
S2 = Sheep blood 2
S3 = Sheep blood 3

Conclusion

From the results, it can be concluded that

- Species-specific primer cytochrome b gene is useful for the differentiation of domestic ruminant species.

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