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Identification of species and sex of domestic animals by PCR based molecular marker

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Abstract
This study is aimed to utilize Polymerase Chain Reaction (PCR) techniques for species and sex identification in domestic animals. By targeting specific genetic markers, namely the Cytochrome b gene for species identification and the SRY gene for sex determination, along with the GAPDH gene as a positive control, the study successfully differentiated between various domestic ruminant species and determined the sex of individual animals. Results revealed distinct band patterns corresponding to different species, with band sizes of 106 bp, 163 bp, 232 bp, and 308 bp for buffalo, cattle, goat, and sheep, respectively. Additionally, the duplex PCR of SRY and GAPDH genes exhibited two bands at 122 bp and 218 bp for male individuals, while female individuals displayed a single band at 218 bp. This study concludes that the Cytochrome b gene primer effectively identifies ruminant species, while the duplex PCR of SRY and GAPDH genes accurately determines the sex of individual animals. These findings underscore the utility of PCR techniques in facilitating precise species and sex identification in domestic animals.

Keywords: Sex of domestic animals, PCR, GAPDH

Introduction
India is blessed with abundant biodiversity, boasting around 400 different mammal species (Sahajpal *et al.*, 2009) [6]. While laws like the Wildlife Protection Act of 1972 safeguard many wild animals, illegal trading and transportation of domestic animals for body their parts like meat, horns, and fur continue to threaten their existence. This illegal activity not only disrupts ecosystems but also endangers other plant and animal species.

Aside from biodiversity conservation, everyday issues like fraudulent meat mixing and illegal cow slaughter persist (Panwar *et al.*, 2015; Sangthong *et al.*, 2021; Apparao *et al.*, 1994) [4, 7, 1]. Such practices not only affect public health and the economy but also hold religious significance.

Crimes against animals are on the rise, leading to the declaration of many species as endangered. After such crimes, biological evidence like blood, skin, or bone is often left behind. However, identifying the species and sex of these samples accurately becomes challenging once they start decomposing. Conventional methods relying on gross anatomical features often fall short in providing accurate identification, making it difficult to prosecute offenders.

To address this challenge, molecular biology techniques offer promising solutions. Molecular genetics can help to develop species-specific and sex-specific genetic markers, aiding in the accurate identification of animals. Techniques like Polymerase Chain Reaction (PCR) and DNA fingerprinting have shown potential in species identification.

Given the need for reliable methods, this study aims to develop PCR-based molecular markers for species and sex identification of domestic animals like buffalo, cattle, sheep, and goats. These markers will enable accurate confirmation of species and sex, contributing to better animal crime investigation and conservation efforts.

Materials and Methods
For the present work, blood samples of six individual animals of each species of either sex i.e.

cattle, buffalo, sheep, and goat were collected in 2 ml of K3EDTA tubes. These collected samples were kept in a refrigerator 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, it was ensured that the OD (Optical density) 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) [7] 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

And for sex, a pair of SRY and GAPDH primers as reported by Prashant *et al.* 2008 [5] i.e. SRY-1F (5'-CGA AGA CGA AAG KTG GCT CT-3' and SRY-1R (5'-TGT GCC TCC TCA AAG AAT GG-3') and GAPDH-1F (5'-CCA ACG TGT CTG TTG TGG ATC TGA-3') and GAPDH-1R (5'-GAG CTT GAC AAA GTG GTC GTT GAG-3') was targeted to produce an amplicon successfully for duplex PCR showing band at 122 bp and 218 bp respectively.

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: Cyclic Condition for Amplification of Species-Specific Marker

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

Table 2: Cyclic Condition for Amplification of Sex-Specific Marker

Stages		Temperature (°C)	Time
Stage 1	Initial denaturation	94 °C	5 min
Stage 2 (35 cycle)	Denaturation	94 °C	30 sec
	Annealing	60 °C	20 sec
	Extension	72 °C	20 sec
Stage 3	Final Extension	72 °C	10 min

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) [7]. On the other hand, despite using the Cytochrome b gene as a primer, Jain *et al.* (2007) [2] 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) [2] 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.

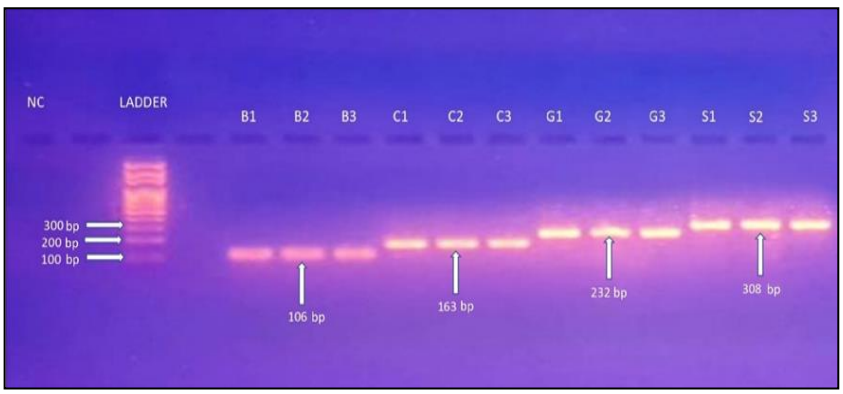

Fig 1: Amplification of species-specific Cytochrome b *gene* of all the species under study from the blood sample.

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

During the current study, it came to light that all male ruminants expressed a single band of the SRY gene at 122 bp, whereas female ruminants did not. Similarly, GAPDH exhibited a band at 218 bp in all samples, despite sex (male or female). The current study found that female ruminants were lacking in any band in the SRY gene. This might be linked to the fact that female ruminants lack amplification of the Y segment of the SRY gene, which could lead to incorrect interpretation of data. The findings of the current study are in correlation with those of Wenfa Lu *et al.* (2006) [8] and Lei Shi *et al.* (2007) [3]. They indicated that the SRY gene is exclusively relevant in determining male samples. As a result, GAPDH, a housekeeping gene predominant in all mammals, was used to detect the band, however, it was discovered that GAPDH detected a band at 218 bp in both males and females. This suggests the GAPDH in simplex PCR failed to differentiate between male and female. Based on the inability of the Y segment to amplify in the SRY gene to distinguish among female ruminants and the inadequacy of GAPDH to discriminate between male and female ruminants, multiplex PCR was very essential. Acknowledging the benefit of GAPDH, which is known for having no association with the amplification of Y-specific target sequences, it was utilised in combination with the SRY gene for duplex PCR. The duplex PCR revealed a band for the SRY gene at 122 bp in male ruminants, while GAPDH represented a band at 218 bp in all ruminants, regardless of sex. The current study's findings revealed that male ruminants have two bands while female ruminants have one. Prashant *et al.* (2008) [5] reported a similar investigation with duplex PCR.

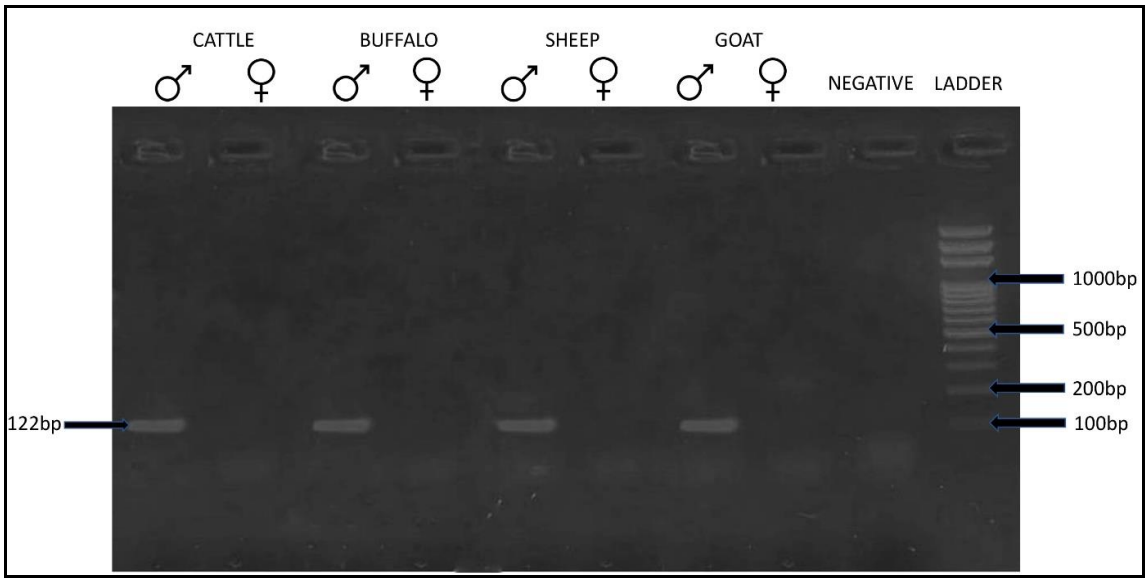

Fig 2: Simplex PCR assay. SRY product (122 bp) is amplified from the Y-specific sequence.

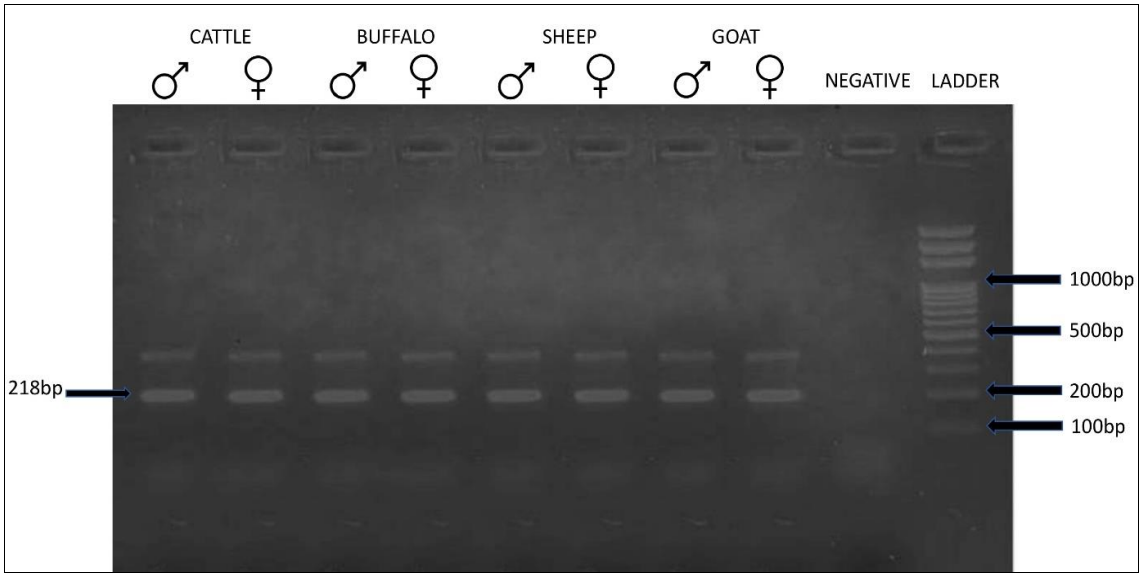

Fig 3: Simplex PCR assay. The GAPDH product (218 bp) is amplified from the X-specific and Y-specific sequence.

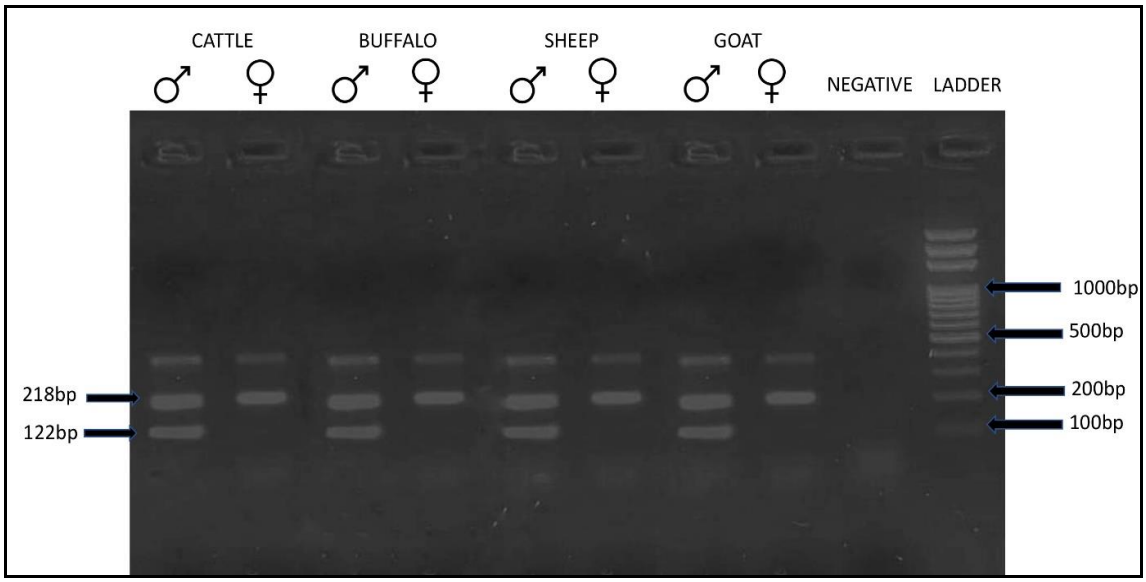

Fig 4: Duplex PCR assay. The GAPDH product (218 bp) is amplified from the X-specific sequence, whereas SRY product (122 bp) is amplified from the Y-specific sequence.

Conclusion

In conclusion, our study investigated the Cytochrome b gene and SRY gene across multiple ruminant species. Regarding Cytochrome b gene amplification, distinct band patterns were observed for each species, consistent with previous studies. However, discrepancies were noted in comparison to some prior findings, likely attributed to primer sequence variations. Additionally, our examination of the SRY gene revealed its specificity in identifying male ruminants, with GAPDH proving less discriminatory between sexes. Thus, multiplex PCR, combining SRY and GAPDH, emerged as a crucial tool for accurate sex determination. Our results underscore the importance of primer design and the need for appropriate molecular techniques to ensure reliable genetic analyses in ruminants.

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