

PCR-BASED MOLECULAR IDENTIFICATION OF SPECIES AND SEX IN DOMESTIC RUMINANTS USING *CYTOCHROME B*, *SRY* AND *GAPDH* MARKERS

Piyush Bhole¹, Rupali Y Charjan^{2*}, Ayesha Boddupalli³, Rathod Rohit Kailasrao⁴, Naina Singh⁵, Nidhi Panicker⁶, Anjali Marutirao Sonphule⁷, Sushil Digambar Hadoltikar⁸

¹Assistant Professor, Department of Veterinary Anatomy and Histology, SGSVVP Lokmangal Veterinary College, Solapur, Maharashtra, India. piyushbhole900@gmail.com

² Professor, Department of Veterinary Anatomy and Histology, Nagpur Veterinary College, Nagpur, Maharashtra, India. email id: rupalicharjan@mafsu.ac.in

³Assistant Professor, Department of Veterinary Anatomy and Histology, Siksha 'O' Anusandhan Deemed to be University, Bhubaneswar, Odisha, India

⁴Ph. D Scholar, Department of Livestock Products Technology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, UP, India

⁵Veterinary Officer Veterinary Clinic, Jamod, Tal. Jalgaon Jamod, Buldhana, Maharashtra, India

⁶Scientist (AICRP on PHET), Department of Veterinary Public Health, Mumbai Veterinary College, Maharashtra, India

⁷Assistant Commissioner of Animal Husbandry, Taluka Veterinary Clinic, Khamgaon, Dist. Buldhana, Maharashtra, India

⁸Medical Superintendent, Subdistrict Hospital, Manchar, Dist Pune, Maharashtra, India

*Corresponding Author: Rupali Y Charjan, rupalicharjan@mafsu.ac.in

ABSTRACT:

Accurate identification of animal species and sex is important in veterinary forensic investigations, wildlife-related cases, meat authentication and the examination of biological evidence. Conventional identification based on morphological characteristics may become difficult when samples are fragmented, degraded or putrefied. The present study evaluated polymerase chain reaction (PCR)-based molecular markers for identification of species and sex from blood samples of domestic ruminants. Blood samples were collected from 48 animals comprising six males and six females each of buffalo (*Bubalus bubalis*), cattle (*Bos indicus*), goat (*Capra hircus*) and sheep (*Ovis aries*). Genomic DNA was isolated using a silica membrane-based commercial extraction kit and assessed for concentration and purity using a spectrophotometer. Species identification was performed using species-specific forward primers targeting the mitochondrial cytochrome b gene in combination with a common reverse primer. Sex identification was performed by amplification of the male-specific *SRY* gene, while *GAPDH* was used as an autosomal amplification control. A duplex PCR combining *SRY* and *GAPDH* was subsequently evaluated. Species-specific PCR generated distinct amplicons of 106 bp in buffalo, 163 bp in cattle, 232 bp in goat and 308 bp in sheep. The *SRY* assay produced a 122-bp fragment in male animals, whereas no *SRY*-specific amplification was observed in females. *GAPDH* produced a 218-bp product in both sexes and was therefore useful as an internal amplification control. Duplex PCR generated two products of 122 and 218 bp in males and only the 218-bp product in females. The findings demonstrate that species-specific cytochrome b PCR can distinguish the four domestic ruminant species examined, while the combined *SRY*-*GAPDH* assay provides a practical molecular approach for sex determination. These assays may be useful in veterinary forensic investigations and other applications requiring molecular identification of domestic ruminant biological samples.

KEYWORDS: Domestic ruminants, PCR, Cytochrome b, *SRY*, *GAPDH*, DNA profiling

INTRODUCTION

Accurate identification of animal-derived biological material is an important component of veterinary forensic science, animal conservation, meat authentication and legal investigations. Biological specimens such as blood, tissue, hair, skin, muscle and bone may be recovered from crime scenes or from materials submitted for examination. Establishing the species and sex associated with such evidence can provide valuable information for investigation and interpretation of the circumstances surrounding a case.

Traditional approaches to species identification have generally relied on morphological, anatomical, histological or immunological characteristics. Although these methods can be useful when an intact specimen is available, their reliability may decrease when the recovered material is small, fragmented, damaged or decomposed. In forensic circumstances, biological material may undergo putrefaction before reaching the laboratory, making identification based solely on gross anatomical characteristics difficult. The source material for the present investigation specifically notes the difficulty of obtaining definitive species and sex identification from small bone fragments and putrefied material using conventional approaches.

Immunological methods, including agar gel diffusion, passive haemagglutination, immuno-electrophoresis, counter-immunoelectrophoresis and enzyme-linked immunosorbent assays, have historically been applied to animal species identification. However, cross-reactivity between closely related species may limit their discriminatory capacity. Molecular genetic approaches overcome several of these limitations because they allow species-specific nucleotide sequences to be detected directly. The uploaded study identified PCR, RAPD, RFLP,

microsatellite analysis, AFLP and DNA sequencing among the molecular approaches that have been applied to species identification.

Among molecular markers, mitochondrial DNA has particular value for species identification. Mitochondrial genes occur in multiple copies per cell and contain sequence differences that can be exploited for discrimination among closely related taxa. The mitochondrial cytochrome b gene has consequently been used in several PCR-based approaches for animal species identification. Previous research has demonstrated that cytochrome b-based PCR can produce characteristic amplicons for different domestic ruminant species. Sangthong *et al.*, (2021), for example, developed a multiplex assay targeting cytochrome b that produced products of 106, 163, 232 and 308 bp for buffalo, cattle, goat and sheep, respectively.

Other investigators have used alternative mitochondrial regions or PCR-RFLP strategies. Karabasanavar *et al.*, (2011), for instance, developed a buffalo-specific PCR assay targeting the mitochondrial D-loop and obtained a 534-bp buffalo-specific product. Similarly, Panwar *et al.*, (2015) reported goat and sheep-specific products of 157 and 331 bp, respectively, using a cytochrome b-based duplex PCR approach. These differences demonstrate that PCR product size depends on the target region and primer design and should therefore be interpreted in relation to the particular assay employed.

In addition to species identification, determination of sex can be an important component of veterinary forensic examination. The SRY gene, located on the Y chromosome, provides a molecular target for identifying male individuals. However, failure to amplify an SRY fragment cannot automatically be interpreted as evidence of a female sample because PCR failure may occur as a consequence of inadequate DNA quality or amplification conditions. A positive amplification control is therefore desirable.

Prashant *et al.*, (2008) developed a duplex PCR assay combining the male-specific SRY gene with the autosomal GAPDH gene for sex determination in six bovine species. Their assay generated a 122-bp SRY product and a 218-bp GAPDH product, allowing males to be distinguished by the presence of both bands and females by the presence of the GAPDH band alone. The authors reported successful application of the approach across several bovid species.

The present study was therefore undertaken to evaluate PCR-based molecular markers for the identification of four commonly encountered domestic ruminant species - buffalo, cattle, goat and sheep - and to determine sex using SRY and GAPDH markers. The study was designed with two principal objectives: (1) molecular identification of domestic ruminant species from blood samples and (2) molecular determination of sex from blood-derived genomic DNA.

MATERIALS AND METHODS

2.1 Experimental animals and sample collection

The investigation was conducted using blood samples obtained from 48 domestic ruminants. The experimental material comprised six males and six females from each of four species: buffalo (*Bubalus bubalis*), cattle (*Bos indicus*), goat (*Capra hircus*) and sheep (*Ovis aries*). Thus, each species was represented by 12 animals, with an equal distribution of males and females.

Blood was collected into K3EDTA-containing tubes to prevent coagulation. Following collection, the tubes were gently inverted three to four times to facilitate mixing of the blood with the anticoagulant. Samples were maintained at -20°C until genomic DNA extraction.

2.2 Reagents and equipment

The principal reagents used included nuclease-free water, PCR master mix, Tris-EDTA buffer, agarose and oligonucleotide primers. Major equipment included a centrifuge, thermocycler, heating block, vortex mixer, microwave oven, electrophoresis apparatus, UV transilluminator, gel documentation system and refrigerator.

2.3 Genomic DNA extraction

Genomic DNA was isolated from blood using the DNeasy Blood and Tissue Kit (Qiagen, Germany), following the manufacturer's protocol. Briefly, 20 μL of proteinase K was added to a microcentrifuge tube, followed by 50–100 μL of anticoagulated blood. The volume was adjusted to 220 μL using phosphate-buffered saline. Subsequently, 200 μL of buffer AL was added, mixed thoroughly and incubated at 56°C for 10 min.

Following incubation, 200 μL of 96–100% ethanol was added. The resulting lysate was transferred to a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 min. The flow-through was discarded. The column was subsequently washed with 500 μL of buffer AW1 and centrifuged at 8000 rpm for 1 min, followed by washing with 500 μL of buffer AW2 and centrifugation at 14,000 rpm for 3 min.

The column was transferred to a clean microcentrifuge tube, and DNA was eluted using 200 μL of buffer AE after a 1-min incubation at room temperature. The eluate was recovered by centrifugation at 8000 rpm for 1 min and stored at -20°C until further analysis.

2.4 DNA quantification and purity assessment

DNA concentration and purity were assessed using an Eppendorf Biospectrometer/NanoDrop spectrophotometer. A 2-μL aliquot of extracted DNA was used for measurement. DNA samples with an expected A260/A280 ratio of approximately 1.7–1.9 and concentration above 50 ng/μL were considered suitable for subsequent PCR analysis.

2.5 Primer selection

Published primer pairs were used for species and sex identification. For species identification, species-specific forward primers targeting the mitochondrial cytochrome b gene of buffalo, cattle, goat and sheep were used in combination with a common reverse primer, following Sangthong *et al.*, (2021). The primer sequences recorded in the experimental material were as follows:

Species/marker	Sequence (5'–3')
Buffalo-specific forward primer	CATACATCCAAACAACGAAGTATG
Cattle-specific forward primer	TAGGAGGAGTACTAGCCCTAGCCT
Goat-specific forward primer	CCTCACATTAAACCTGAGTGGTAT
Sheep-specific forward primer	CATGCTACTAGTACTATTACGCCT
Common reverse primer	CTGGYTGKCCTCCAATYCATG

The SRY and GAPDH primers were adopted from the method of Prashant *et al.*, (2008).

Marker	Primer	Sequence (5'–3')
SRY	FORWARD	CGAAGACGAAAGKTGGCTCT
SRY	REVERSE	TGTGCCTCCTCAAAGAATGG
GAPDH	FORWARD	CCAACGTGTCTGTTGTGGATCTGA
GAPDH	REVERSE	GAGCTTGACAAAGTGGT CGTTGAG

2.6 Preparation of species-specific PCR

Working primer concentration was prepared from the supplied lyophilized primers. Stock solutions were prepared at 100 pmol/μL and subsequently diluted to obtain working solutions of 10 pmol/μL.

Each species-specific PCR reaction had a final volume of 25 μL consisting of 2 μL DNA template, 1 μL species-specific forward primer, 1 μL common reverse primer, 12.5 μL master mix and 8.5 μL nuclease-free water.

The amplification program consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 61°C for 30s and extension at 72°C for 30s. A final extension was performed at 72°C for 30s.

2.7 SRY and GAPDH PCR

For individual SRY or GAPDH amplification, a 25-μL reaction consisted of 2 μL DNA template, 1 μL forward primer, 1 μL reverse primer, 12.5 μL master mix and 8.5 μL nuclease-free water.

2.8 Duplex PCR for sex determination

For simultaneous amplification of SRY and GAPDH, each 25-μL reaction contained 2 μL DNA template, 1 μL SRY forward primer, 1 μL GAPDH forward primer, 1 μL SRY reverse primer, 1 μL GAPDH reverse primer, 12.5 μL master mix and 6.5 μL nuclease-free water.

The duplex amplification program consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 20 s and extension at 72°C for 20 s. A final extension was performed at 72°C for 10 min. These cycling conditions correspond closely with the previously published SRY-GAPDH duplex approach.

2.9 Agarose gel electrophoresis

PCR products were analyzed by horizontal submarine agarose gel electrophoresis using 2% (w/v) agarose. A 1×Tris-acetate-EDTA buffer was used for preparation and electrophoresis. The gel was prepared by dissolving 4 g agarose in 200 mL of 1× buffer and heating until completely dissolved. Ethidium bromide was added according to the original experimental protocol.

Approximately 8 μL of PCR product was loaded into the gel along with a negative control and 100-bp DNA ladder. Electrophoresis was performed at 110 V for 70 min. Following electrophoresis, amplified DNA fragments were visualized using a gel documentation system and UV transillumination.

RESULTS

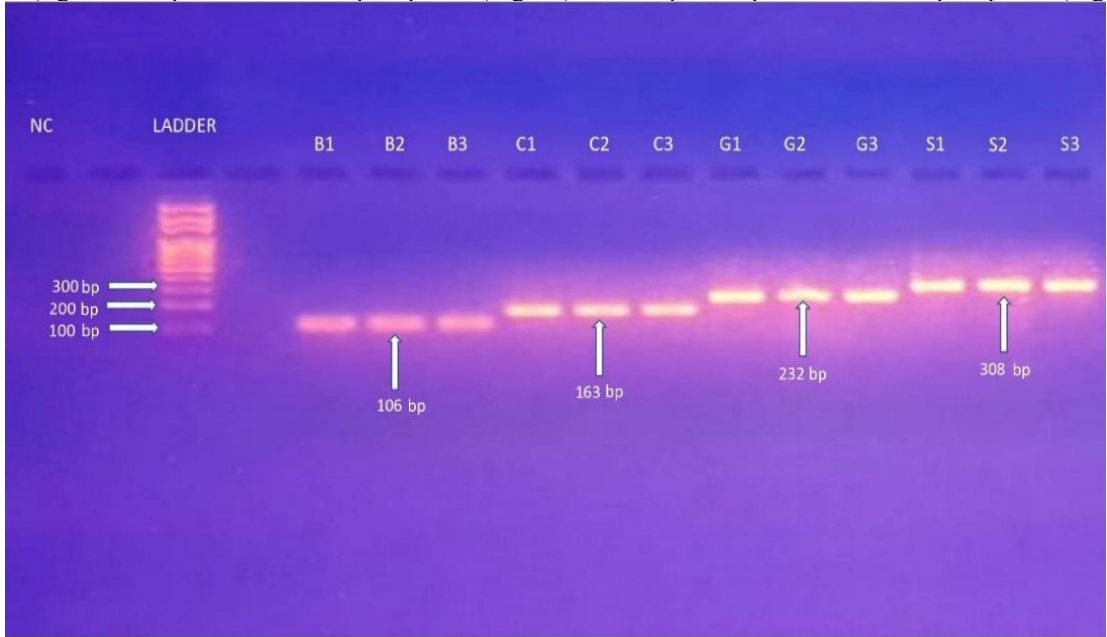
DNA extraction and quality assessment

Genomic DNA was successfully extracted from blood samples obtained from all four domestic ruminant species using the commercial silica membrane-based extraction procedure. DNA concentration and purity were assessed spectrophotometrically before PCR amplification. Samples meeting the required concentration and purity criteria were used for downstream molecular analysis.

3.1 Species identification by cytochrome b-based PCR

Species-specific PCR generated characteristic DNA fragments for each of the four domestic ruminant species examined (Fig 3.1). Buffalo DNA produced a 106-bp amplicon (Fig 3.2), cattle DNA produced a 163-bp amplicon

(Fig 3.3), goat DNA produced a 232-bp amplicon (Fig 3.4) and sheep DNA produced a 308-bp amplicon (Fig 3.5).



NC = Negative control

Ladder = 100 bp ladder

B1 = Buffalo blood 1

B2 = Buffalo blood 2

B3 = Buffalo blood 3

C1 = Cattle blood 1

C2 = Cattle blood 2

C3 = Cattle blood 3

G1 = Goat blood 1

G2 = Goat blood 2

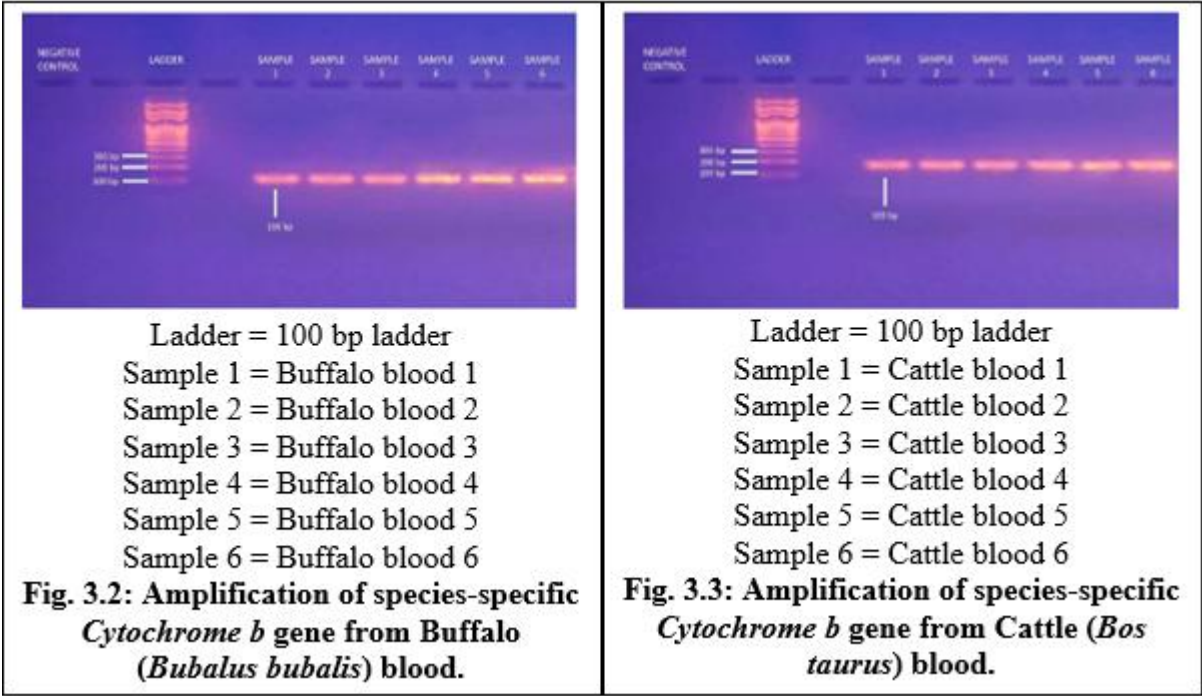
G3 = Goat blood 3

S1 = Sheep blood 1

S2 = Sheep blood 2

S3 = Sheep blood 3

Fig. 3.1: Amplification of species-specific Cytochrome b gene of all the species under study from the blood sample.



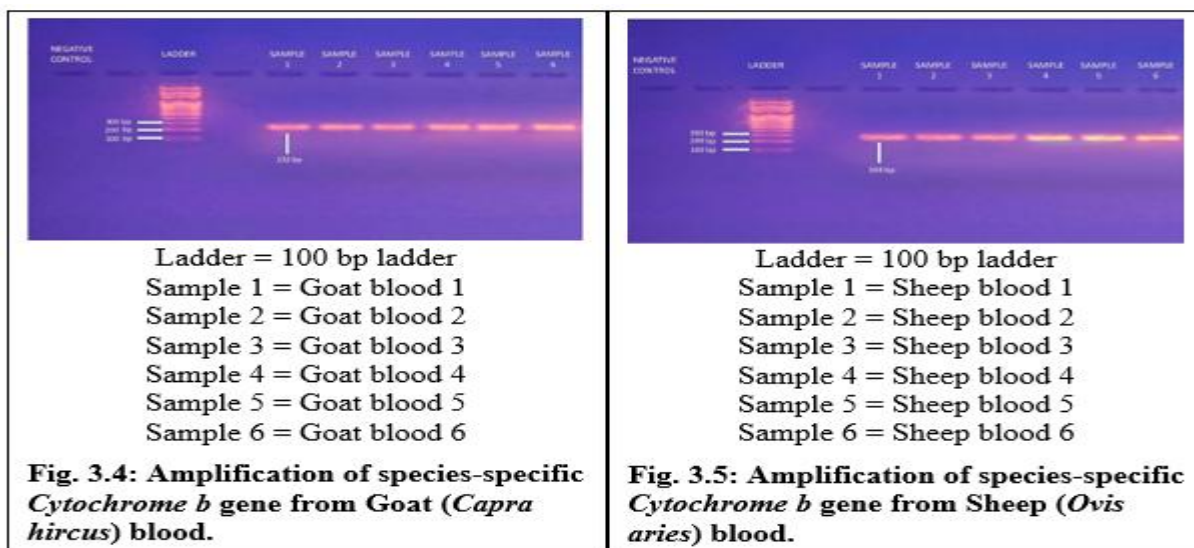


Table 3.1. Species-specific PCR products obtained from domestic ruminant blood samples

Species	Scientific name	Target marker	Observed amplicon size
Buffalo	<i>Bubalus bubalis</i>	<i>Cytochrome b</i>	106 bp
Cattle	<i>Bos indicus</i>	<i>Cytochrome b</i>	163 bp
Goat	<i>Capra hircus</i>	<i>Cytochrome b</i>	232 bp
Sheep	<i>Ovis aries</i>	<i>Cytochrome b</i>	308 bp

The buffalo-specific product was observed at 106 bp. This result corresponded to the amplicon size reported by Sangthong *et al.*, (2021) using their species-specific cytochrome b assay.

Cattle samples produced a 163-bp fragment. This was also consistent with the 163-bp cattle-specific product reported by Sangthong *et al.*, (2021).

Goat DNA produced a 232-bp product. The observed fragment size corresponded with the goat-specific amplicon described by Sangthong *et al.*, (2021), although other studies using different targets and primer systems have reported different sizes.

The sheep-specific reaction produced a 308-bp fragment, again corresponding with the result reported by Sangthong *et al.*, (2021).

3.2 Sex identification using SRY PCR

The conventional SRY PCR generated a 122-bp product in male animals. No SRY-specific product was observed in female samples. Thus, the presence of the 122-bp SRY fragment served as the molecular indicator of male sex in the examined animals (Fig 3.6).

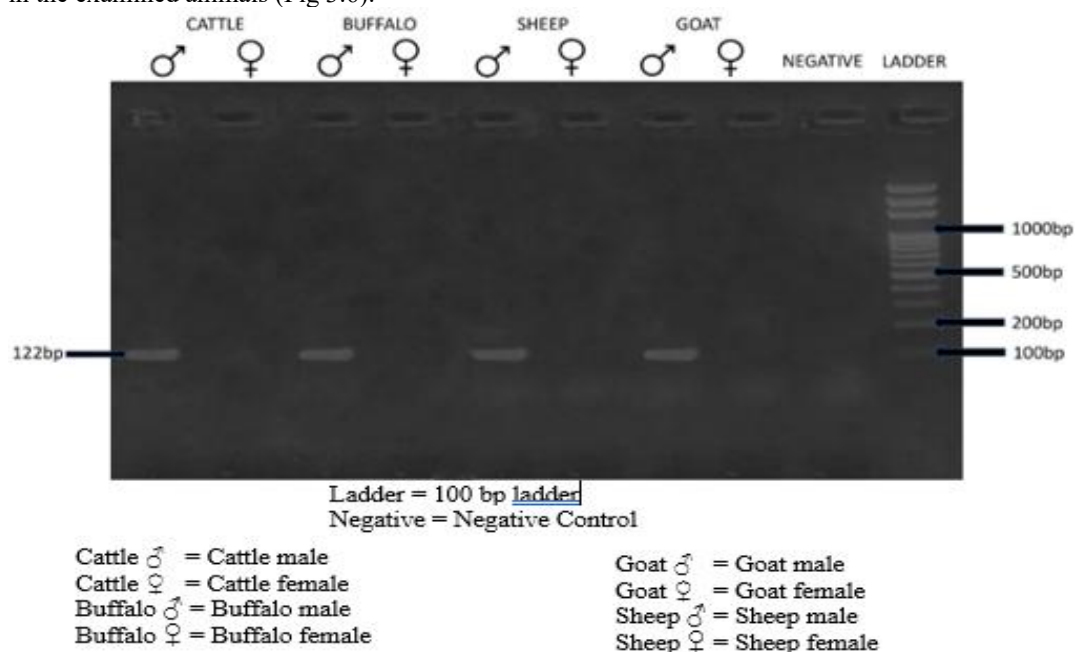


Fig. 3.6: Amplification of SRY gene for identification of sex from blood sample of domestic ruminants.

3.3 GAPDH amplification

Amplification of the GAPDH gene generated a 218-bp fragment in both male and female animals. Because GAPDH is autosomal and is not restricted to one sex, the presence of this product demonstrated that amplifiable genomic DNA was present in the PCR reaction (Fig 3.7).

An additional approximately 400-bp nonspecific product was observed in GAPDH PCR. The same additional product was also observed in both sexes. The uploaded study notes that a similar nonspecific band had previously been reported in bovid DNA samples by Prashant *et al.*, (2008).

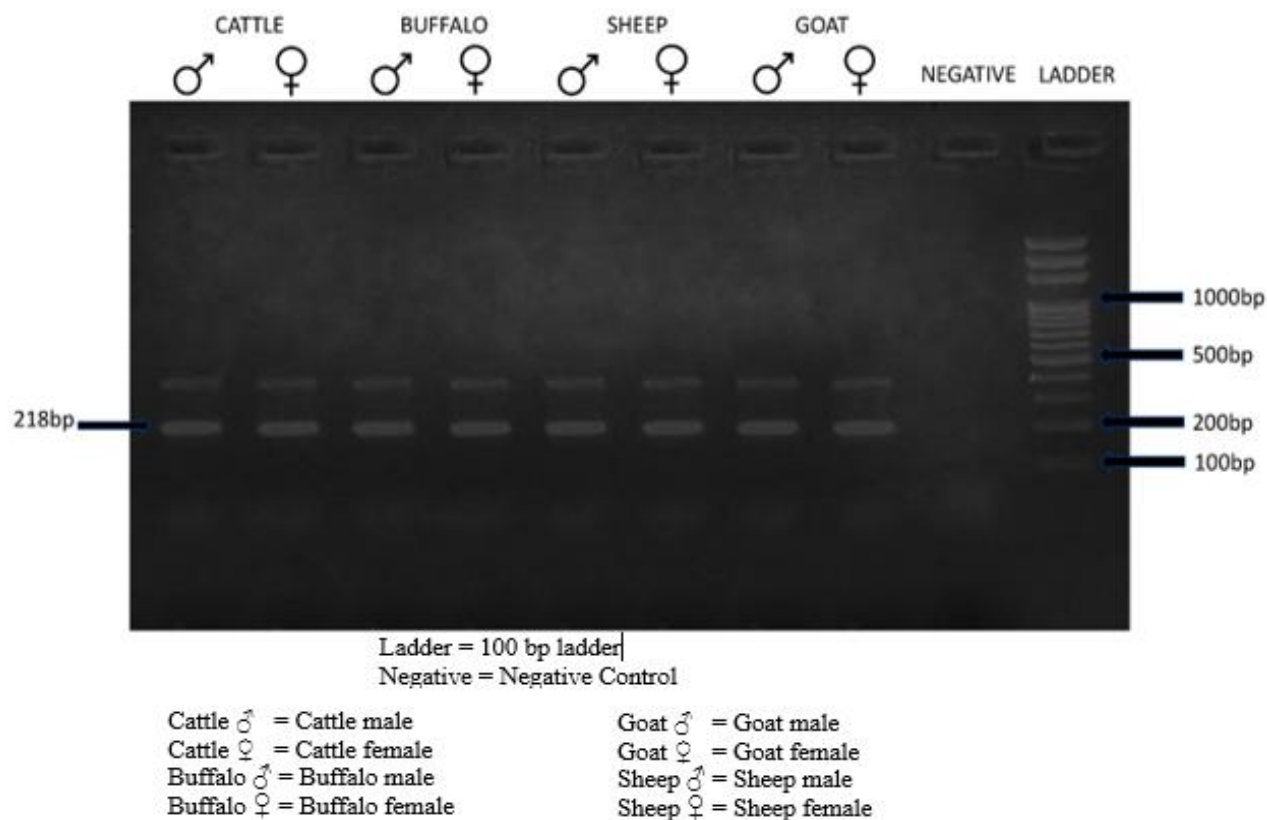


Fig. 3.7: Amplification of GAPDH gene for identification of sex from blood sample of domestic ruminants.

3.4 Duplex PCR for sex determination

The combined SRY-GAPDH duplex PCR produced a characteristic sex-dependent banding pattern. Male animals exhibited two bands corresponding to SRY at 122 bp and GAPDH at 218 bp, whereas females exhibited only the 218-bp GAPDH band. The additional approximately 400-bp product was also observed as a nonspecific band (Fig 3.8).

Table 2. Banding pattern obtained in SRY-GAPDH duplex PCR

Sex	SRY product	GAPDH product	Interpretation
Male	122 bp	218 bp	Male
Female	Not detected	218 bp	Female
Both sexes	~400 bp*	—	Nonspecific product

The approximately 400-bp band was reported as an additional nonspecific product in the experimental observations.

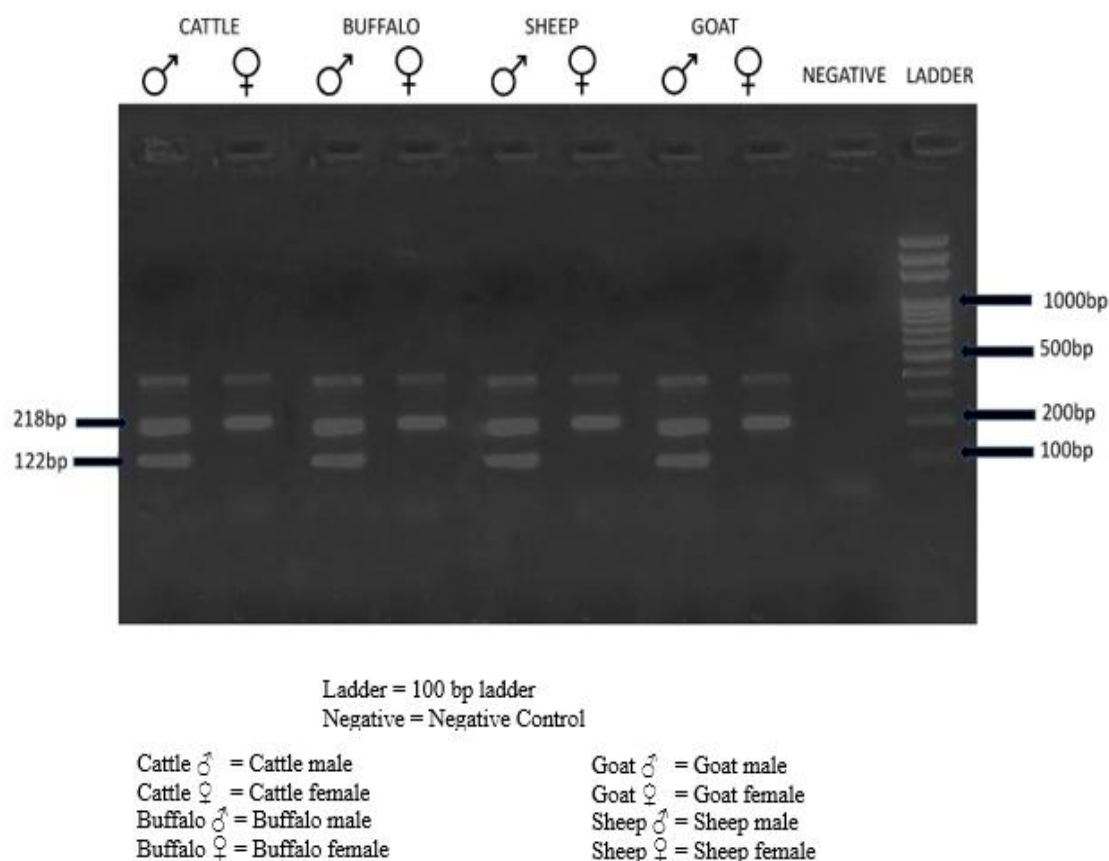


Fig. 3.8: Amplification of SRY and GAPDH gene for identification of sex from blood sample of domestic ruminants.

Table 3. Summary of molecular markers used in the study

Application	Marker	Expected/observed product	Diagnostic interpretation
Buffalo identification	<i>Cytochrome b</i>	106 bp	Buffalo
Cattle identification	<i>Cytochrome b</i>	163 bp	Cattle
Goat identification	<i>Cytochrome b</i>	232 bp	Goat
Sheep identification	<i>Cytochrome b</i>	308 bp	Sheep
Male identification	SRY	122 bp	Male
Genomic amplification control	GAPDH	218 bp	Amplifiable DNA in both sexes
Sex determination	SRY + GAPDH	122 + 218 bp	Male
Sex determination	SRY + GAPDH	218 bp	Female

DISCUSSION

The present investigation demonstrated that PCR-based molecular markers can provide species-specific and sex-specific information from blood-derived genomic DNA of domestic ruminants. The four species examined generated distinct cytochrome b-associated amplification products, while the SRY-GAPDH duplex generated a sex-dependent banding pattern.

4.1 Species identification

The use of species-specific PCR provides an important advantage over morphological identification when biological evidence is limited or degraded. The present study produced characteristic fragments of 106 bp for buffalo, 163 bp for cattle, 232 bp for goat and 308 bp for sheep. These products correspond exactly with the species-specific fragment sizes reported by Sangthong *et al.*, (2021), who developed a multiplex PCR assay based on cytochrome b sequences for buffalo, cattle, goat and sheep.

The agreement between the present observations and those reported by Sangthong *et al.*, supports the utility of these species-specific targets for distinguishing the four domestic ruminant species. In the present study, the individual species-specific primers were applied to blood-derived DNA rather than to processed meat or dairy products, thereby demonstrating their applicability to another type of biological sample.

The mitochondrial cytochrome b gene has been widely used for molecular species identification because sequence

variation between taxa can provide discriminatory markers. The use of short diagnostic fragments can also be advantageous when working with biological samples in which DNA may be partially degraded.

However, amplicon size should not be considered an intrinsic characteristic of a species. It is a property of the particular primer-target combination. This is demonstrated by the variation among previously published studies. Karabasanavar *et al.*, (2011), for example, targeted the mitochondrial D-loop region for buffalo identification and obtained a 534-bp buffalo-specific product rather than the 106-bp product observed with the present cytochrome b assay.

Likewise, Panwar *et al.*, (2015) reported 157- and 331-bp products for goat and sheep, respectively, using a different cytochrome b-based primer system. Chikuni *et al.*, (1994) also demonstrated that PCR targeting a sheep satellite DNA sequence could distinguish sheep and goat from several other animal species, illustrating that different genetic targets can provide different diagnostic fragment patterns.

Therefore, the value of the present assay lies not merely in the numerical size of the PCR products but in the reproducible association between each specific primer-target system and its corresponding species.

4.2 Buffalo identification

The buffalo-specific assay generated a 106-bp fragment. This finding was identical to the buffalo-specific fragment reported by Sangthong *et al.*, (2021).

The difference between this 106-bp product and the 534-bp product reported by Karabasanavar *et al.*, (2011) is readily explained by the use of different molecular targets and primer systems. Karabasanavar *et al.*, targeted the mitochondrial D-loop and designed a buffalo-specific assay that generated a 534-bp product. Thus, the two results should not be regarded as contradictory; they represent independent diagnostic PCR systems.

4.3 Cattle identification

A 163-bp cattle-specific fragment was observed in the present study. This was consistent with the result reported by Sangthong *et al.*, (2021). The distinction of cattle from buffalo is particularly relevant in forensic and food-authentication contexts because both species may be represented in biological or meat-derived evidence.

The ability to obtain a discrete species-specific fragment provides a relatively straightforward interpretation when an appropriate positive control and molecular size standard are included.

4.4 Goat and sheep identification

The goat and sheep assays generated 232- and 308-bp products, respectively. The separation between these two products provides a clear molecular distinction between the two closely related domestic ruminants examined in the present work.

Previous studies have used different genetic regions to discriminate goat and sheep. Panwar *et al.*, (2015), using a different cytochrome b-based assay, reported 157 bp for goat and 331 bp for sheep. Chikuni *et al.*, (1994) used a sheep satellite-DNA target and demonstrated differentiation through PCR and subsequent restriction analysis. These findings emphasize that molecular species identification can be achieved through several marker systems, with the choice depending on assay design, sample type and intended application.

4.5 Molecular sex determination

The SRY assay produced a 122-bp fragment exclusively in male animals. The SRY gene is located on the Y chromosome and therefore provides a useful molecular marker for male identification in mammals. The present observation agrees with the duplex PCR approach described by Prashant *et al.*, (2008), who reported a 122-bp SRY product in males and a 218-bp GAPDH product in both males and females.

A key advantage of incorporating GAPDH into the assay is that absence of an SRY band alone does not have to be interpreted immediately as evidence of female sex. A failed PCR reaction could also produce an SRY-negative result. Detection of the autosomal GAPDH product demonstrates that amplifiable genomic DNA was present. Consequently, a female pattern can be interpreted as GAPDH-positive/SRY-negative rather than simply SRY-negative.

The duplex assay in the present study therefore generated a straightforward pattern: males produced two products 122 bp and 218 bp whereas females produced the 218-bp GAPDH product alone. This pattern is consistent with the previously reported SRY-GAPDH duplex assay.

4.6 Nonspecific amplification

An approximately 400-bp additional band was observed in the GAPDH and duplex PCR reactions in the present study. Because this band occurred in both males and females and did not correspond to the diagnostic products of interest, it was interpreted as nonspecific amplification. The same type of approximately 400-bp additional band has been reported by Prashant *et al.*, (2008) in buffalo, cattle and other bovids.

Although the presence of a nonspecific band may complicate gel interpretation, it did not prevent identification of the diagnostic 122-bp SRY and 218-bp GAPDH products in the present study.

4.7 Forensic significance

The results have potential relevance to veterinary forensic investigations in which biological material must be assigned to a particular domestic ruminant species. The original study was motivated by the difficulty of identifying decomposed or limited biological samples using gross anatomical characteristics alone.

PCR-based approaches can provide molecular evidence that complements conventional veterinary anatomical examination. Species identification can potentially assist investigations involving illegal animal slaughter, meat substitution, animal-derived biological evidence and other circumstances in which species attribution is required. Similarly, molecular sex determination may be useful when morphological sex characteristics are unavailable or unsuitable for examination. The SRY-GAPDH duplex approach provides an additional molecular tool because it simultaneously detects a male-specific target and an autosomal amplification control.

Nevertheless, the present study should be interpreted within its experimental scope. The work involved known blood samples from four domestic ruminant species. It did not evaluate highly degraded forensic specimens, mixed-species samples, processed tissues, environmental samples or samples from a broader range of closely related taxa. Therefore, further validation would be necessary before applying the assay as a standalone forensic test to unknown casework material.

CONCLUSION

The present study demonstrated the applicability of PCR-based molecular markers for identification of four domestic ruminant species and determination of sex from blood-derived genomic DNA.

Species-specific amplification generated distinct products of 106 bp for buffalo, 163 bp for cattle, 232 bp for goat and 308 bp for sheep, providing molecular differentiation among the four species. The SRY assay produced a 122-bp male-specific fragment, whereas GAPDH generated a 218-bp product in both males and females.

The SRY-GAPDH duplex assay consequently generated a characteristic two-band pattern of 122 and 218 bp in males and a single 218-bp band in females. The presence of GAPDH as an autosomal amplification control strengthens interpretation of an SRY-negative result.

Overall, the findings support the use of species-specific cytochrome b PCR for domestic ruminant identification and the SRY-GAPDH duplex system for molecular sex determination. These approaches may have applications in veterinary forensic investigations, animal-derived biological evidence examination, meat authentication and related diagnostic situations. However, additional validation using larger sample populations, blind testing and degraded or mixed biological specimens would strengthen their forensic applicability.

REFERENCES

1. Abu Chikuni, K., Tabata, T., Kosugiyama, M., Monma, M., & Saito, M. (1994). Polymerase chain reaction assay for detection of sheep and goat meats. *Meat Science*, 37(3), 337–345. [https://doi.org/10.1016/0309-1740\(94\)90051-5](https://doi.org/10.1016/0309-1740(94)90051-5)
2. Karabasanavar, N. S., Singh, S. P., Umapathi, V., Girish, P. S., Shebannavar, S. N., & Kumar, D. (2011). Authentication of carabeef (water buffalo, *Bubalus bubalis*) using highly specific polymerase chain reaction. *European Food Research and Technology*, 233, 985–989. <https://doi.org/10.1007/s00217-011-1583-9>
3. Panwar, N., Gahlot, G. C., Gahlot, K., Ashraf, M., & Singh, A. (2015). Rapid identification of goat (*Capra hircus*) and sheep (*Ovis aries*) species in raw meat using duplex PCR assay. *Indian Journal of Animal Research*, 49(4), 537–541. <https://doi.org/10.5958/0976-0555.2015.00077.1>
4. Prashant, Gour, D. S., Dubey, P. P., Jain, A., Gupta, S. C., Joshi, B. K., & Kumar, D. (2008). Sex determination in 6 bovid species by duplex PCR. *Journal of Applied Genetics*, 49(4), 379–381. <https://doi.org/10.1007/BF03195637>
5. Sangthong, D., Suwannarat, S., Samipak, S., & Sangthong, P. (2021). Multiplex PCR assay for species identification of meat and dairy products from buffalo (*Bubalus bubalis*), cattle (*Bos indicus* and *Bos taurus*), goat (*Capra hircus*), and sheep (*Ovis aries*). *International Food Research Journal*, 28(4), 716–725. <https://doi.org/10.47836/ifrj.28.4.08>