



## Authentication of sheep (*Ovis aries*) and goat (*Capra hircus*) meat species using species-specific polymerase chain reaction

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Received: 3 February 2016; Accepted: 4 April 2016

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### ABSTRACT

Small ruminants (sheep and goat) immensely contribute to the Indian meat food basket; yet regional preference for either sheep or goat meat varies. Owing to the differences in the market prices, these two closely resembling species meats are often misrepresented interchangeably. Prevention of such fraudulent practices requires authentic and sensitive speciation tools. A novel mitochondrial D-loop based species-specific polymerase chain reaction (PCR) technique was developed for the precise detection of origin of sheep and goat meats. Species-specific primers (SSP-G and SSP-S) developed for PCR in this study amplify 229 and 425 base pairs products specific to goat and sheep species. An additional heteroplasmic PCR product of size 350 bp was evident only in sheep. Primers designed in this study were checked for the absence of cross-species amplification in closely related animal species and was successfully applied for the authentication of a forensic case. Our results indicated prerogative applications of the novel species-specific PCR for the authentic identification of origin of sheep and goat species thereby aiding in the prevention of the menace of misrepresentation of meats.

**Key words:** Adulteration, Goat, Meat, Mitochondrial DNA, PCR, Sheep

Testing meat authenticity is of paramount importance to verify product's label claim and prevent the practice of fraudulent meat substitution which is objectionable on health (allergy) and economic grounds. Such cheating of consumers for economic reasons is punishable under the court of law. Hence, it requires precise and unambiguous identification of origin of species so as to ensure fair trade, quality assurance and public health protection.

PCR or its variants are the most widely used techniques for the identification of meat animal species owing to their specificity, sensitivity, accuracy and precision. DNA is the most widely used target due to higher stability and omnipresence in every cell. The DNA based animal speciation techniques offer several advantages over the conventional protein-based techniques like isoelectric focusing (King 1984), electrophoresis (Bhilegaonkar *et al.* 1990), counter immune electrophoresis (Sherikar *et al.* 1993), chemometric analysis (Ioannis and Van Maria 2003) and enzyme linked immune-sorbent assay (Martin *et al.* 1991). Hence, DNA based techniques such as PCR or its variants like arbitrarily primed-PCR (Desmais *et al.* 1998),

DNA hybridization (Buntjer *et al.* 1999), random amplified polymorphic DNA fingerprinting (Saez *et al.* 2004), restriction fragment length polymorphism (Girish *et al.* 2005, 2007), sequence analysis (Girish *et al.* 2004, 2009), species specific PCR (Girish *et al.* 2013) and PCR-single strand conformation polymorphisms (Tejedor *et al.* 2006). The species-specific PCR in particular offers several advantages over other DNA based techniques in terms of its simplicity and economy of operation. Novel PCR technique intended for detection of meat misrepresentation was developed in the present study for the authentic identification of sheep and goat meats using species-specific primers.

### MATERIALS AND METHODS

**Sample collection:** Meat samples of cattle (*Bos indicus*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*) and goat (*Capra hircus*) were collected from municipal slaughterhouse. Forensic sample was received from a local body in Andhra Pradesh. All the samples were stored at –20°C in a deep freezer until further analysis.

**Genomic DNA extraction:** Extraction of DNA from meat was carried out as per Chikuni *et al.* (1990). Isolated DNA was checked for quality, purity and concentration spectrophotometrically ( $OD_{260/280} \sim 1.76$  to 1.89) and through agarose gel electrophoresis.

**Designing of species-specific primers:** Nucleotide sequences of sheep and goat were downloaded from the

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Table 1. Sheep and goat species-specific primers used for PCR

Species	Primer	Sequence	Amplicon
Sheep	SSP- SF	Forward-5'-AAA AGC ACA ACC ATC CAC CC-3'	425 bp
	SSP- SR	Reverse-5'-GTC AAG CAG TTC AATGCC CTA T-3'	
Goat	SSP- GF	Forward-5'-GGA AGA AGC CAT AGC CTC AC-3'	229 bp
	SSP- GR	Reverse-5'-AGT TGG GTT AGG ATTGGG AT-3'	

National Centre for Biotechnology Information (NCBI) database. Species-specific primers were designed targeting mitochondrial D loop region using DNA STAR<sup>®</sup> software and custom synthesized as shown in Table 1.

*PCR amplification of sheep and goat specific mitochondrial D loop gene:* Gradient PCR amplification was standardized differing in annealing temperatures between 54° to 60°C. Optimum amplification was evident at 56°C, hence it was fixed as the annealing temperature. Ready master mix was mixed with 15 picomoles each of forward and reverse primers and 50 ng of template DNA. Thermocycler program was standardized with initial denaturation for 5 min at 94°C followed by 30 cycles of amplification (denaturation for 1 minute at 94°C, primer annealing for 1 min at 56°C and extension for 1 min at 72°C) and final extension for 5 min at 72°C. Amplicons were checked by agarose (2%) gel electrophoresis with ethidium bromide staining.

## RESULTS AND DISCUSSION

*Species-specific primers for the identification of sheep and goat species:* Novel species-specific primer pairs yielded species-specific PCR amplicons of size 229 and 425 base pairs using SSP-GF/GR and SSP-SF/SR primers in goat and sheep species, respectively. Annealing temperature gradient revealed optimum amplification at 56°C (Fig. 1) for both the species. An additional amplicon of size 350 bp was evident in sheep and it is not such an uncommon phenomenon in sheep; due to 'heteroplasmy' more than 2 types (copy number) of mitochondrial DNA

occur in sheep leading to additional PCR products (Malisa *et al.* 2006, Karabasanavar *et al.* 2011a). However, as the amplicon 425 bp size was the prominent, it was taken as a diagnostic signal in the present study.

Species-specific PCR offers authentic identification of meat species compared to the other speciation techniques such as PCR-RFLP (restriction fragment length polymorphism) as reported by Zimmerman *et al.* (1998) and Girish *et al.* (2005). It is time saving, economical and easy way of performing and interpreting the test results. Compared to the forensically important nucleotide sequencing (FINS) technique as reported previously (Girish *et al.* 2004, 2009), species-specific PCR is better as the former requires nucleotide sequencing and sequence alignment using bioinformatics tools a conserved consensus region; and also there is an additional cost of sequencing, software and expertise.

Advent of real time PCR offered quantitative analysis to the forensic science. Resultantly, it was also applied for speciation of small ruminants (Cassard 2001); however, owing to costly reagents its affordability for routine laboratory analysis is unwarranted. Further, as the species-specific PCR just reads presence or absence of a respective band after electrophoresis, the purpose of animal speciation is served. Similarly, real time PCR won't offer exceptionally superior sensitivity over the conventional end-point PCR.

Compared to the nuclear sequences, mitochondrial targets offer several advantages; hence, are used as targets for species detection, viz. cyt-b gene, cyt oxidase I gene, rRNA (12S, 16S, 18S, 28S), tRNAs (tRNAGlu, tRNALys-ATPases), ND5 etc (Karabasanavar *et al.* 2011b). Of which, targeting of D-loop region was found to be promising as it offers inter-species and inter-genus comparisons (Lockwood *et al.* 1993, Karabasanavar *et al.* 2010, Kumar *et al.* 2011) that arise due to its unique replication, mutation and evolutionary changes.

*Validation of novel PCR repeatability:* Cross-species amplification of newly designed primers was checked by testing closely related species. Single PCR product of 229 bp was observed only in goat using goat-specific PCR (Fig. 2A) and its repeatability was checked by including cattle, buffalo, sheep and goat species DNA. Except for 425 bp PCR product in the sheep, no amplification was found in cattle, buffalo and goat using sheep-specific primers (Fig. 2B).

*Application of PCR for forensic sample:* To solve forensic cases, samples received from the local government authorities were analyzed in the laboratory for the authentication of label claims or misrepresentation of sheep

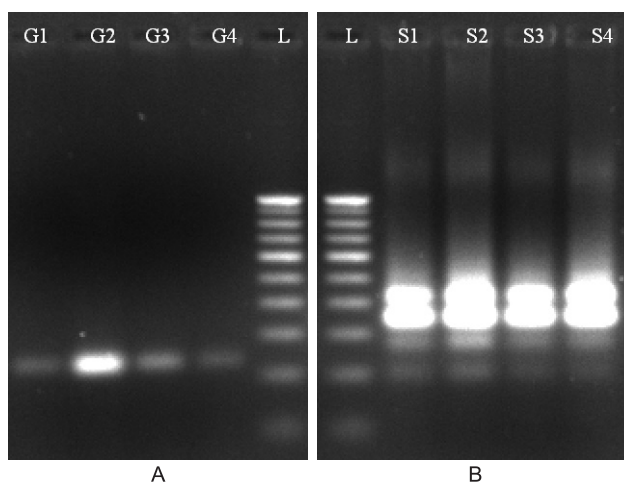


Fig. 1. Gradient PCR amplification of DNA from mutton using species-specific primers. Lanes 1, 2, 3, 4 indicate annealing temperature of 54°, 56°, 58° and 60°C.

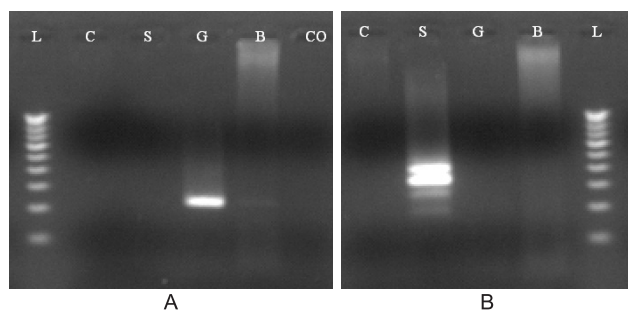


Fig. 2. Targeted amplification of mitochondrial D-loop region using novel species-specific PCR (gel image reveals absence of amplification in other related species). [A: goat, B: sheep]. Lane C, cattle; S, sheep; G, goat; B, buffalo; L, 100 bp ladder.

meat. Template DNA was prepared from samples and novel species-specific PCR technique was applied. Upon amplification, products of size 425 and 350 bp (Fig. 3) were observed using SSP-SF/SR primers; however, no amplification was observed using SSP-GF/GR primers. Based on these results, forensic sample was confirmed as sheep species. To countercheck the results, PCR amplification and nucleotide sequencing of mitochondrial 12S rRNA gene was performed as described previously (Girish *et al.* 2004). Results of the novel species-specific PCR and forensically important nucleotide sequencing (FINS) were found to be exactly matching. The results of the species-specific PCR were validated based on its comparison with another DNA based technique i.e. FINS.

Novel species-specific PCR technique was developed for the authentic identification of goat and sheep species meat, which has applications in solving forensic and meat adulteration issues. Species-specific primers designed targeting mitochondrial D loop region yielded PCR products of size 229 and 425 bp specific to goat and sheep species.

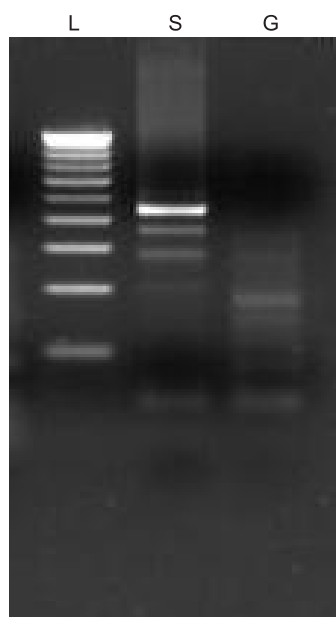


Fig. 3. Sheep-specific PCR for detecting unknown forensic sample. L, 100 bp ladder; S, sheep; G, goat.

Developed PCR technique was found reproducible and convincing for the purpose of sheep and goat species identification.

#### ACKNOWLEDGEMENT

Authors would like to sincerely thank the Director, ICAR - National Research Centre on Meat, Hyderabad for providing financial assistance to carry out this study.

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