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Original Research

Species Identification of Biological Samples Derived from Wild Ungulate Species by Forensically Informative Nucleotide Sequencing of Mitochondrial 12S rRNA Gene

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Abstract

Authentic identification of wild animal species is warranted to prevent poaching and illegal trade of the wild life. Wild ungulates are one of the most vulnerable species for poaching which necessitates authentic techniques for species identification of biological samples derived from them. In this work, biological samples (tissues or blood) were collected from five available ungulate species viz., black buck (Antilope cervicapra), barking deer (Muntiacus muntjak), mouse deer (Moschiola meminna), nilgai (Boselaphus tragocamelus) and spotted deer (Axis axis). The DNA was extracted from the samples by following standard protocol and the extracted DNA was subjected to polymerase chain reaction using universal primers targeting mitochondrial 12S rRNA gene which yielded about 450 bp amplicon in all species. Amplicons were sequenced and aligned using bioinformatic tools. Results indicated that the species identification of wild ungulates can be achieved by aligning the nucleotide sequence data with the already available sequences in the gene bank of National Centre for Biotechnology Information which will give the list of species in the order of their similarity (highest to lowest). Highest identity was deduced as the closest species. Observations made in this study endorse the application of Forensically Informative Nucleotide Sequence (FINS) for the forensic detection of biological samples derived from wild ungulate species and hence it holds potential to contribute in the effort to conserve wild ungulate.

Keywords: DNA, FINS, Mitochondria, PCR,12S rRNA gene, Speciation

Introduction

Reducing forest cover, increasing urbanization, continued wildlife poaching and man-animal conflicts have tremendously impacted the survival of wildlife. The demographic changes such as Corona Virus Infections Disease (COVID) - 2019 lockdowns have significantly increased poaching of wild ungulates at their habitats (Badola, 2020). India is a treasure of 35 species of ungulates; of which, 25 are protected under Wildlife Protection Act of 1972 (Daniel,1991). Many factors are contributing to declining numbers of the wild ungulates: expanding human population, increasing man-animal conflicts, declining quantity and quality of land, water scarcity during critical times, decreasing forest cover, etc. Of all these, illegal poaching is a major contributing factor. Since wild ungulates play balancing role in the nature in terms of their crucial function in food chains of forest ecosystems, their decreasing number is a serious concern requiring conservation activities so as to preserve the biodiversity. Herbivores act as a food source to carnivores and human induced population disturbance in herbivores directly contributes to the vulnerability of carnivores (Proffitt *et al*., 2013; Xiao *et al*., 2018). Ungulates are polyphyletic hoofed quadruped mammals that play important role in forest ecosystems. In the Indian context, wild herbivores such as barking deer (*Muntiacus muntjak)*, black buck (*Antilope cervicapra)*, Indian gaur (*Bos gaurus)*, mouse deer (*Moschiola meminna)*, nilgai (*Boselaphus tragocamelus)* and spotted deer (*Axis axis)* are protected under Wildlife Protection Act, 1972.

Spotted deer (*Axis axis*) population is drastically declining at an estimated extinction rate of 45% over the last 50 years in the Indian sub-continent (Karanth *et al*., 2010). However, at some places it acts as an invasive species and outweigh in number especially in protected areas (Mohanty *et al*., 2016). India's 123 protected areas and forest tracts nurture spotted deer (Sankar and Acharya, 2004). Nevertheless, spotted deer are killed illegally by humans for flesh, skin and antlers. Black buck (*Antilope cervicapra*) is widespread across Indian sub-continent; it mostly occurs in the Tarai region (Bashistha *et al.,* 2012). Although this ecological indicator once inhabited the entire Indian subcontinent, but now its number is decreasing due to habitat destruction and poaching (Roberts, 1992). Barking deer (*Muntiacus muntjak*) or the Indian muntjak is one of the smallest deer belonging to Cervidae family. Even though muntjaks are listed under least concern category by the International Union for Conservation of Nature and Natural Resources (IUCN); their depleting number is of concern that occurs due to habitat destruction and poaching (Timmins *et al*., 2016). The small size in particular makes them highly vulnerable to hunting or poaching for meat and skin.

Mouse deer (*Moschiola meminna*) is a small nocturnal solitary artiodactyl that inhabit densely covered forests. Mouse deer is also known as Indian chevrotain and it lacks antlers (evolutionarily primitive). It is distributed across the India mostly in the Deccan peninsula including eastern and Western Ghats, Central India, Gangetic plains (except West Bengal) and the Tarai region bordering Nepal. Encroachment of forest due to anthropogenic activities and poaching has adversely affected this species (Duckworth and Timmins, 2015). Indian gaur (*Bos gaurus*) has been categorized as vulnerable by the IUCN. Drastic decline in its population due to various factors including hunting have initiated conservation activities in India (Sankar *et al*., 2013; Choudhury, 2002). Gaur is mostly hunted in several Indian states for meat and horns. Nilgai (*Boselaphus tragocamelus)* is also known as blue bull; this largest Antelope is widely distributed in India. Nilgai is perceived as an agricultural pest in India since it depredates crops (Goyal and Rajpurohit 1999; IUCN, 2006). As per Indian Hindu doctrine, Nilgai is perceived sacred and treated on par with the cow; its hunting or harassing refrained (Prater, 1980). However, instances of its hunting for flesh arise as it has resemblance with that of beef.

Authentic species identification of poached species helps in the wildlife protection and conservation initiatives. Use of different molecular markers has evolved as a powerful tool in species identification. Previously, techniques such as liquid chromatography (Dratch *et al.,* 1996), immunoassay (Ubelaker *et al*., 2004), electrophoresis (Abraham *et al*., 2001), etc were for species identification of biological samples. Nevertheless, in the recent years, DNA based techniques are increasingly been used due to their higher specificity and stability. Mitochondrial DNA targets have been conclusively proven for species identification (Irwin *et al*., 1991; Hayashi *et al*., 1985; Gupta *et al*., 2015); this offers exploitation of variations in the mitochondrial DNA for the purpose of discrimination of closely related species that offers inter-species sequence variations. Mitochondrial DNA has been used extensively due to high copy number of mitochondria in the cell. Mitochondria follow clonal inheritance, only dam contributes mitochondria, genome does not undergo recombination and hence its genetic material is transferred to the next generation unchanged (Galtier *et al*., 2009). Further, mitochondrial genome accumulates high percentage of neutral mutations that aid in animal species identification. Each mitochondrion contains 2-6 circular DNA molecules, has

16,500 bp size and codes several genes (Gardner and Snustad, 1984). Several DNA based techniques have been developed for species identification of ungulates: species specific PCR (Paul *et al*., 2019), Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR – RFLP) (Rajput *et al*., 2013; Siddappa *et al*., 2013; Gupta *et al*., 2008) and real time PCR (Davitkov *et al*., 2017). In the present study, conserved region of mitochondrial 12S rRNA sequence was amplified using universal primers and the amplicon was sequenced and analyzed to identify the biological samples derived from wild ungulates.

Materials and Methods

Sample Collection

Muscle tissue and blood samples of wild ungulates were collected from the Nehru Zoological Park, Hyderabad, India. Muscle tissues were collected from carcasses undergoing postmortem examination. Samples were collected from black buck (*Antilope cervicapra*), barking deer (*Muntiacus muntjak*), mouse deer (*Moschiola meminna*), nilgai (*Boselaphus tragocamelus*) and spotted deer (*Axis axis*). Permission to collect the samples was obtained from the Principal Chief Secretary, Department of forest, Telangana state, India as well as the Director and Curator of Nehru Zoological Park, Hyderabad, India. Samples were transported under chilled condition and stored at -20 °C until further analysis.

Extraction of DNA from Muscle Tissues

Tissue DNA was extracted using the method of Chikuni *et al.* (1994). Briefly, tissue was digested with 10 volumes of Tris HCl (10 mM, pH 8.0) containing 100 mM ethylene diamine tetra acetate, 0.5% sodium dodecyl sulphate and 0.1 mg/ml of proteinase K for 3 h at 50 °C. Resultant lysate was incubated with RNAase (50 mg/ml) for 1 h at 37 °C and extracted with equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1, v/v) followed by another extraction with equal volume of chloroform. Extracted DNA was precipitated using ethanol and ammonium acetate (1 M); finally dissolved in Tris-EDTA buffer (1X, 10 mM Tris HCl, pH 7.4; 0.1 mM EDTA). Extracted DNA was stored at -20 °C until further use.

Extraction of DNA from Blood

The DNA was extracted from blood samples using a commercial kit (GCC Biotech, West Bengal, India) as per manufacturer's instructions. Integrity of isolated DNA was checked by agarose (1%) gel electrophoresis in Tris Acetate EDTA buffer (40 mM tris-acetate, 2 mM EDTA, pH 8.0) in accordance with Sambrook and Russel (2001). Concentration of DNA was measured using spectrophotometer (Make: Nanodrop, USA).

Polymerase Chain Reaction Using Universal Primers

Conserved partial sequence of mitochondrial 12S rRNA gene was amplified using the universal primers targeting mitochondrial 12S rRNA gene as described by Kocher *et al*. (1989) and Girish *et al*. (2004). Nucleotide sequence of the primers: Forward - 5′ CAA ACT GGG ATT AGA TAC CCA CTA 3′ and Reverse - 5′ GAG GGT GAC GGG CGG TGT GT 3′. The PCR reaction mix of 25 µl contained Tris-HCl (10 mM, pH 8.3), KCl (50 mM), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM), 10 pmol each forward and reverse primers, 60 ng of template DNA and 1U Taq DNA polymerase (GCC Biotech, West Bengal, India.) and nuclease free water. The PCR was carried out in thermal cycler (Make: Applied Biosystems, USA) with the thermal cycling conditions; initial denaturation at 94 °C for 5 min; followed by 30 cycles of denaturation (95 °C, 30 s), annealing (60, 62, 60, 55, 55, 60 and 60 °C for mouse deer, barking deer, black buck, nilgai, spotted deer, sheep and goat, respectively) for 1 min and extension (72 °C for 1 min) with the final extension at 72 °C for 5 min. The PCR products were electrophoresed over 1 % agarose gel and visualized through gel documentation system.

Nucleotide Sequencing and Sequence Analysis

The PCR product of each species was custom sequenced (IRA Biotech, Hyderabad) and analyzed through Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST hit showing the highest identity was deduced as the closest species. Further, multiple sequence alignment of sequences was carried out using Clustal W algorithm (DNA STAR

software, Lasergene).

Results and Discussion

Polymerase Chain Reaction Amplification of Mitochondrial 12S rRNA Gene

Quality of DNA isolated from biological samples was found suitable for PCR as assessed by agarose gel (0.8%) electrophoresis which indicated single and high molecular weight DNA without any shearing. DNA samples showing DNA 260/280 ratio between 1.8 and 2.2 were considered for further processing. The PCR amplification using the universal primers targeting mitochondrial 12S rRNA gene was undertaken in the following species: spotted deer, black buck, barking deer, nilgai, mouse deer, Indian gaur, sheep and goat. PCR amplification yielded amplicons of about 450 bp size in all the species (Figure 1).

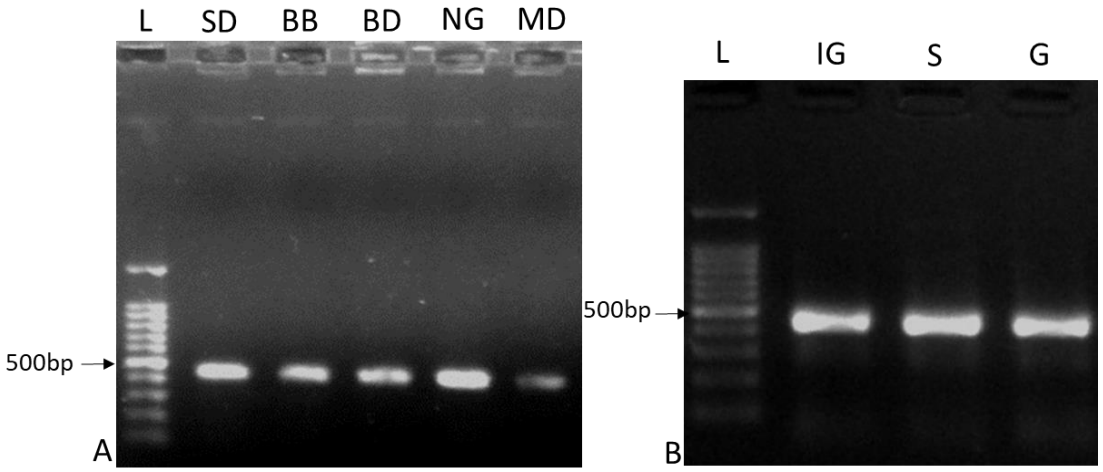

Figure 1 (A and B): Polymerase chain reaction amplification of mitochondrial12S rRNA gene run on 1% agarose gel. L: 100 bp DNA ladder; SD: Spotted deer; BB: Black buck; BD: Barking deer; NG: Nilgai; MD: Mouse deer; IG: Indian gaur; S: Sheep; G: Goat

Universal primers have advantage of common primer pairs for all the wild animals which makes the assay simple and easy to execute. But the amplicon size slightly vary from species to species. Variations in the amplicon size are encountered due to evolutionary changes in the species genome. Girish *et al*. (2004) also amplified mitochondrial 12S rRNA gene in domestic meat animals *viz.*, cattle (*Bos indicus*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*) and goat (*Capra hircus*) and reported amplicon size of about 456 bp in all the species. Panicker *et al*. (2019) amplified mitochondrial 12S rRNA gene in about thirteen different wild animal species including deer and reported amplicon of the size of about 450 bp. Nevertheless, irrespective of the minor variations in amplicon size, sequencing of the amplicon can be undertaken in all the amplicons to enable species identification of the sample.

Mitochondrial 12S rRNA gene has been targeted as a marker for the species identification of meat in this work. Mitochondrial genome of animals is a better target than the nuclear genome for studies involving inter species polymorphism because of its lack of introns, limited exposure to recombination and haploid mode of inheritance (Saccone *et al.* 1999). Hence, mitochondrial genes have been commonly targeted for species identification of wild animals (Paul *et al*., 2019; Siddappa *et al*., 2013; Panicker *et al*., 2019)

Sequencing of the Amplicons and Sequence Analysis

PCR amplicons were sequenced with the help of commercial sequencing facility. Nucleotide sequence analysis of mitochondrial 12S rRNA gene sequences of spotted deer (*Axis axis*), black buck (*Antilope cervicapra*), barking deer (*Muntiacus muntjak*), mouse deer (*Moschiola meminna*), Indian gaur (*Bos gaurus*) and nilgai (*Boselaphus tragocamelus*) was undertaken using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/entrez) which showed highest nucleotide homology with the respective species. Details of the nucleotide sequences used in this study and their accession numbers are given in Table 1.

Table 1: Details of wild ungulate species and the accession numbers of nucleotide sequences used in the present study

S. No.	Common name	Species	Accession number	Reference
1	Black Buck	*Antilope cervicapra*	MT071791	*
2	Barking Deer	*Muntiacus muntjak*	MT125867	*
3	Sheep	*Ovis aries*	AJ490504	Girish *et al*. 2004
4	Nilgai	*Boselaphus tragocamelus*	MT125898	*
5	Mouse Deer	*Moschiola meminna*	MT125896	*
6	Indian gaur	*Bos gaurus*	MT125881	*
7	Spotted deer	*Axis axis*	MT125918	*
8	Goat	*Capra hircus*	AJ490503	Girish *et al*. 2004

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Sequence alignment was done using CLUSTAL W algorithm *Megalign* program (Laser Gene, DNA STAR Software) for determining similarity and divergence (Figure 2).

```
     - - - - A T A A T T G T A T A A A C A A  Majority
                         10                  20
     ---------------------------------------
1    - - - - - - - - - - T T A T A A A C A A  Barking deer #.seq
1    - - - - - - - - - - - - - - - - - - - A  Black buck Seq #.seq
1    - - A A A T A A T T A C A G A A A C A A  Goat #.seq
1    - - - - - - - A T T G T A T A A A C A A  Indian gaur #.seq
1    - - - - A T A G T T G T - T T A A C A A  Mouse deer #.seq
1    A C A G A T A A T T A T A C A A A C A A  Nilgai #.seq
1    - C A A A T A A T T A T A A G A A C A A  Sheep #.seq
1    A C C A A T A G T T G T A T A A A C A A  Spotted deer Seq #.seq

     A A T T A T T - C G C C A G A G T A C T  Majority
                         30                  40
     ---------------------------------------
11   A A C T A T T - C G C C A G A G T A C T  Barking deer #.seq
2    A A T T A T T T C G C C A G A G C A C T  Black buck Seq #.seq
19   A A T T A T T - C G C C A G A G T A C T  Goat #.seq
14   A A T T A T T T T G C C A G A G T A C T  Indian gaur #.seq
16   A T C T A T T - C G C C A G A G T A C T  Mouse deer #.seq
21   G A T T A T T - C G C C A G A G T A C T  Nilgai #.seq
20   A A T T A T T - C G C C A G A G T A C T  Sheep #.seq
21   A A C T A T T - C G C C A G A G T A C T  Spotted deer Seq #.seq

     A C C G G C A A C A G C T T A A A A C T  Majority
                         50                  60
     ---------------------------------------
30   A C C G G C A A T A G C T T A A A A C T  Barking deer #.seq
22   A C C G G C A A T A G C C T A A A A C T  Black buck Seq #.seq
38   A C C G G C A A C A G C C C G A A A C T  Goat #.seq
34   A C C A G C A A C A G C T T A A A A C T  Indian gaur #.seq
35   A T T A G C C A C A G C T T A A A A C T  Mouse deer #.seq
40   A C C G G C A A C A G C T T A A A A C T  Nilgai #.seq
39   A C C G G C A A C A G C C C G A A A C T  Sheep #.seq
40   A C C G G C A A T A G C T T A A A A C T  Spotted deer Seq #.seq
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```
     C A A A G G A C T T G G C G G T G C T T  Majority
                        70                  80
     ----------------------------------------
50   C A A A G G A C T T G G C G G T G C T T  Barking deer #.seq
42   C A A A G G A C T T G G C G G T G C T T  Black buck Seq #.seq
58   C A A A G G A C T T G G C G G T G C T T  Goat #.seq
54   C A A A G G A C T T G G C G G T G C T T  Indian gaur #.seq
55   C A A A G G A C T T G G C G G T G C T T  Mouse deer #.seq
60   C A A A G G A C T T G G C G G T G C T T  Nilgai #.seq
59   C A A A G G A C T T G G C G G T G C T T  Sheep #.seq
60   C A A A G G A C T T G G C G G T G C T T  Spotted deer Seq #.seq

     T A T A T C C T T C T A G A G G A G C C  Majority
                        90                 100
     ----------------------------------------
70   T A T A C C C T T C T A G A G G A G C C  Barking deer #.seq
62   T A T A T C C T T C T A G A G G A G C C  Black buck Seq #.seq
78   T A T A C C C T T C T A G A G G A G C C  Goat #.seq
74   T A T A T C C T T C T A G A G G A G C C  Indian gaur #.seq
75   T A T A T C C A T C T A G A G G A G C C  Mouse deer #.seq
80   T A T A T C C C T C T A G A G G A G C C  Nilgai #.seq
79   T A T A C C C T T C T A G A G G A G C C  Sheep #.seq
80   T A T A C C C T T C T A G A G G A G C C  Spotted deer Seq #.seq

     T G T T C T A T A A T C G A T A A A C C  Majority
                       110                 120
     ----------------------------------------
90   T G T T C T A T A A T C G A T A A A C C  Barking deer #.seq
82   T G T T C T A T A A T C G A T A A A C C  Black buck Seq #.seq
98   T G T T C T A T A A T C G A T A A A C C  Goat #.seq
94   T G T T C T G T A A T C G A T A A A C C  Indian gaur #.seq
95   T G T T C T A T A A T C G A T A A A C C  Mouse deer #.seq
100  T G T T C T A T A A T C G A T A A A C C  Nilgai #.seq
99   T G T T C T A T A A T C G A T A A A C C  Sheep #.seq
100  T G T T C T A T A A T C G A T A A A C C  Spotted deer Seq #.seq


     C C G A T A A A C C T C A C C A A T C C  Majority
                       130                 140
     ----------------------------------------
110  C C G A T A G A C C T C A C C A T T C C  Barking deer #.seq
102  C C G A T A A A C C T C A C C A A T C C  Black buck Seq #.seq
118  C C G A T A A A C C T C A C C A A T C C  Goat #.seq
114  C C G A T A A A C C T C A C C A G T T C  Indian gaur #.seq
115  C C G A T A A A C C T C A C C A A C C C  Mouse deer #.seq
120  C C G A T A A A C C T C A C C A A T T C  Nilgai #.seq
119  C C G A T A A A C C T C A C C A A T C C  Sheep #.seq
120  C C G A T A A A C C T C A C C A T T C C  Spotted deer Seq #.seq

     T T G C T A A T A C A G T C T A T A T A  Majority
                       150                 160
     ----------------------------------------
130  T C G C T A A T A C A G T C T A T A T A  Barking deer #.seq
122  T T G C T A A T A C A G T C T A T A T A  Black buck Seq #.seq
138  T T G C T A A T A C A G T C T A T A T A  Goat #.seq
134  T T G C T A A T A C A G T C T A T A T A  Indian gaur #.seq
135  T C G C T A A T C C A G T C T A T A T A  Mouse deer #.seq
140  T T G C T A A T T C A G T C T A T A T A  Nilgai #.seq
139  T T G C T A A T A C A G T C T A T A T A  Sheep #.seq
140  T T G C T A A T C C A G T C T A T A T A  Spotted deer Seq #.seq

     C C G C C A T C T T C A G C A A A C C C  Majority
                       170                 180
     ----------------------------------------
150  C C G C C A T C T T C A G C A A A C C C  Barking deer #.seq
142  C C G C C A T C T T C A G C A A A C C C  Black buck Seq #.seq
158  C C G C C A T C T T C A G C A A A C C C  Goat #.seq
154  C C G C C A T C T T C A G C A A A C C C  Indian gaur #.seq
155  C C G C C A T C T T C A G C A A A C C C  Mouse deer #.seq
160  C C G C C A T C T T C A G C A A A C C C  Nilgai #.seq
159  C C G C C A T C T T C A G C A A A C C C  Sheep #.seq
160  C C G C C A T C T T C A G C A A A C C C  Spotted deer Seq #.seq
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    T A A A A A - G G A A C A A A A G T A A  Majority
                        190                 200
170 T A A A A A - G G A G C A A A A G T A A  Barking deer #.seq
162 T G A A A A - G G A A C A A A A G T A A  Black buck Seq #.seq
178 T A A A A A - G G A A C A A A A G T A A  Goat #.seq
174 T A A A A A - G G A A G A A A A G T A A  Indian gaur #.seq
175 T A A A A A - G G A A C A A A A G T A A  Mouse deer #.seq
180 T A A A A A - G G A A T A A A A G T A A  Nilgai #.seq
179 T A A A A A A G G G A C A A A A G T A A  Sheep #.seq
180 T A A A A A - G G T A C A A A A G T A A  Spotted deer Seq #.seq

    G C A C A A T C A T A A T A C A T A A A  Majority
                        210                 220
189 G C G C A A T C A T A G T A C A T A A A  Barking deer #.seq
181 G C A C G A T C A T A A G A C A T A A A  Black buck Seq #.seq
197 G C T C A A T C A C A A C A C A T A A A  Goat #.seq
193 G C A T A A T C A T A A T A C A T A A A  Indian gaur #.seq
194 G C T C A A C C A T T A T A C A T A A A  Mouse deer #.seq
199 G C A C A A T A A T A A C A C A T A A A  Nilgai #.seq
199 G C T C A A T A A T A A C A C A T A A A  Sheep #.seq
199 G C A C A A T C A T A A T A C A T A A A  Spotted deer Seq #.seq

    A A C G T T A G G T C A A G G T G T A A  Majority
                        230                 240
209 A A C G T T A G G T C A A G G T G T A A  Barking deer #.seq
201 A A C G T T A G G T C A A G G T G T A A  Black buck Seq #.seq
217 G A C G T T A G G T C A A G G T G T A A  Goat #.seq
213 A A C G T T A G G T C A A G G T G T A A  Indian gaur #.seq
214 A A C G T T A G G T C A A G G T G T A A  Mouse deer #.seq
219 A A C G T T A G G T C A A G G T G T A A  Nilgai #.seq
219 G A C G T T A G G T C A A G G T G T A A  Sheep #.seq
219 G A C G T T A G G T C A A G G T G T A A  Spotted deer Seq #.seq

    C C T A T G G A A T G G G A A G A A A T  Majority
                        250                 260
229 C C T A T G G A A T G G A A A G A A A T  Barking deer #.seq
221 C C T A T G G A G T G G G A A G A A A T  Black buck Seq #.seq
237 C C C A T G G A A T G G G A A G A A A T  Goat #.seq
233 C C T A T G A A A T G G G A A G A A A T  Indian gaur #.seq
234 C C T A T G G G A T G G G A A G C A A T  Mouse deer #.seq
239 C C T A T G A A A T G G A A A G A A A T  Nilgai #.seq
239 C C T A T G G A G T G G G A A G A A A T  Sheep #.seq
239 C C T A T G G A A T G G A A A G A A A T  Spotted deer Seq #.seq

    G G G C T A C A T T T C T A C C T T A  Majority
                        270                 280
249 G G G C T A C A T T T C T A A T C C A  Barking deer #.seq
241 G G G C T A C A T T T C T A T C T C A  Black buck Seq #.seq
257 G G G C T A C A T T T C T A C C T T A  Goat #.seq
253 G G G C T A C A T T C T C T A C A C T A  Indian gaur #.seq
254 G G G C T A C A T T T T C T A C A T A A  Mouse deer #.seq
259 G G G C T A C A T T T T C T A T A T T A  Nilgai #.seq
259 G G G C T A C A T T T T C T A C C C A A  Sheep #.seq
259 G G G C T A C A T T T T C T A A T A T A  Spotted deer Seq #.seq

    A G A A - A A C T T A A T A C G A A A G  Majority
                        290                 300
269 A G A A C A A C T C A A T A C G A A A A  Barking deer #.seq
261 A G A A - A A C T T A A T A C G A A A G  Black buck Seq #.seq
277 A G A A - A A - T T A A T A C G A A A G  Goat #.seq
273 A G A G - A A C C A A A C A C G A A A G  Indian gaur #.seq
274 T A A G - A A - - - C A C A C G A A A A  Mouse deer #.seq
279 A G A A - A A T C T T A C A C G A A A G  Nilgai #.seq
279 G A A A - A T - T T A A T A C G A A A G  Sheep #.seq
279 A G A A - A A T C C A C T A C G A A A G  Spotted deer Seq #.seq
```

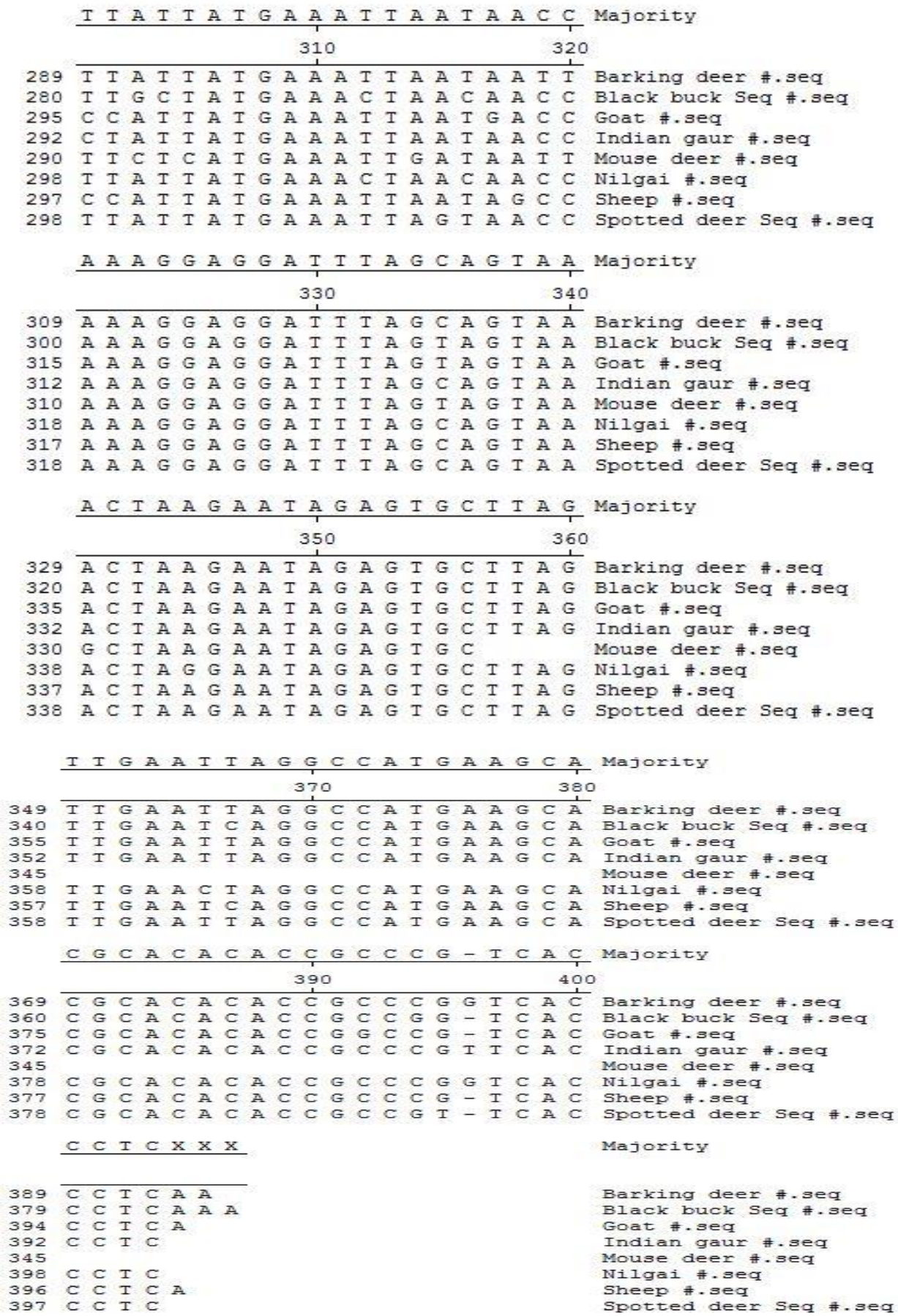

Figure 2: Nucleotide sequence alignment of mitochondrial 12S rRNA gene of different wild animal species. A dot indicates identity with the majority at the given position

Similarity index of 12S rRNA gene showed percent identity and divergence scores for different species adequate to identify species (Table 2). Sequence comparison showed 75.6- 95.5% homology among deer species studied. Among deer species, barking deer showed divergence of 5.3 and 9.4 with closely related species *viz*., spotted deer (*Axis axis*) and sheep (*Ovis aries*). Among wild ungulates, aligning of amplicon sequences using Clustal W tool showed highest similarity (93.9%) between barking deer and spotted deer and lowest (75.6 %) between black buck and mouse deer. Divergence score between different species enables the differentiation of the species of wild ungulates. Identification of species and determination of inter-species relationships is of paramount importance for the discourses in biology, ecology, evolution, systematics, wildlife management, conservation and forensic science (Tobe *et al*., 2010) and FINS can aid in these initiatives.

Table 2: Nucleotide similarity (upper triangle, %) and divergence (lower triangle, %) of mitochondrial 12S rRNA gene of different wild ungulate species

Species name	Barking deer	Black buck	Goat	Indian gaur	Mouse deer	Nilgai	Sheep	Spotted deer
Barking deer	-	89.8	90.9	91.4	76.9	90.4	90.4	93.9
Black buck	8.1	-	92.5	90.9	75.6	91.7	91.9	91.4
Goat	8.8	7	-	90.7	75.6	91.5	95.5	91.7
Indian gaur	8.5	9.1	8.5	-	77	92.9	90.6	92.2
Mouse deer	11.5	13.4	14.2	12.2	-	87.5	87	88.7
Nilgai	9.7	7.8	8.9	7.3	13.9	-	90.3	91.5
Sheep	9.4	7.5	4.7	9.3	14.6	9.8	-	90.8
Spotted deer	5.3	8.1	8.6	7.8	12.3	8.8	9.4	-

Paul *et al*. (2019) developed a species-specific molecular marker for swamp deer and four others coexisting cervids sambar, chital, barking deer and hog deer. These markers showed species-specific band patterns and a high success rate of 88.21% which indicate that the results may not always be unambiguous. Authors also pointed out that subsequent use of these primers to other regions would require testing them with samples from other respective areas. Chances of such false negative reactions are higher in wild life forensics wherein the quantity of sample may be very low and degraded which may not yield DNA of adequate quality. Davitkov *et al*. (2017) undertook PCR amplification of the DNA extracted from the different types of wild life forensic samples and concluded that the classical (end point) PCR method provided results from a total of 119 (92 %) samples, while ten samples were negative (4 feces samples, 3 hair samples, 2 swab samples and 1 pelt sample). All blood and spleen samples were positive. All samples that failed to be amplified by end point PCR were positive using real time PCR (100%). This indicates that false negative reactions are common in PCR assays, especially in difficult samples which are very common in wild life forensics. Pranay *et al*. (2016) checked four published species-specific primers for tiger and leopard for cross amplification in other species and found that three of them cross amplified with other wild animal species and yielded same product size. They concluded that erroneous results in species specific PCR is a possibility, if proper in-silico and lab-based checks are not performed. Due to huge number of wild species involved, it will be practically impossible to check cross amplification in all concerned species. Hence, species specific PCR can jeopardize the investigations involving wild animals with serious repercussions. In FINS based method as the sequence data will be available for verification by any court of law, the results are most reliable. Siddappa *et al*. (2013) reported a PCR – RFLP based method targeting mitochondrial 12S rRNA gene sequence for species identification of mouse deer and its differentiation from the other deer species using restriction enzymes *Rsa*I, *Dde*I, *Bsr*I, and *Bst*SFI. Using multiple enzymes for RFLP adds to the cost and point mutation leading to false negative results are a possibility which needs to be factored in. Hence, it is not advisable to use PCR RFLP based technique as a sole approach for speciation in wild life forensics.

Phylogenetic Analysis Using the Sequence Data

Mitochondrial 12S rRNA gene is a highly conserved gene in animals; hence used as molecular marker for species identification and establishing molecular phylogeny (Arif *et al*., 2009). Mitochondrial 12S rRNA gene sequence of different ungulate species was used to construct phylogenetic tree (Figure 3) in this work; phylogenetic analysis revealed divergent evolution of mouse deer as a separate group from other ungulate species. While, barking deer and spotted deer were found to be evolved from the same ancestor that also resulted in the evolution of black buck

and sheep as well as goat (Nidhi *et al.*, 2013). However, nilgai and Indian gaur were found to evolve from same ancestor. Our results showed that Mouse deer is far related to Barking deer and Spotted deer which is in agreement with the phylogenetic analysis report of Siddappa *et al*., 2013. Phylogenetic relationship of different ungulates reported in this report is in agreement with the report of Kumar *et al*., 2014 who developed phylogenetic tree using the polymorphism in Cytochrome B and 12S rRNA gene. Hebert *et al*., 2003 developed a DNA based method for taxonomic classification of species popularly known as DNA bar coding. This technique is based on sequence analysis of cytochrome C oxidase I (COI) gene for taxonomic profiling of species including species identification of meat. Universal primers used in the present study amplified part of 12S rRNA gene from wide variety of ungulate species. Mouse deer probably evolved very early as primitive ruminant. From phylogenetic analysis it is evident that it evolved early as separate from other deer species family. Mitochondrial 12S rRNA gene was earlier used to identify different species like spotted deer, hog deer and sambar deer (Panicker *et al.*, 2019).

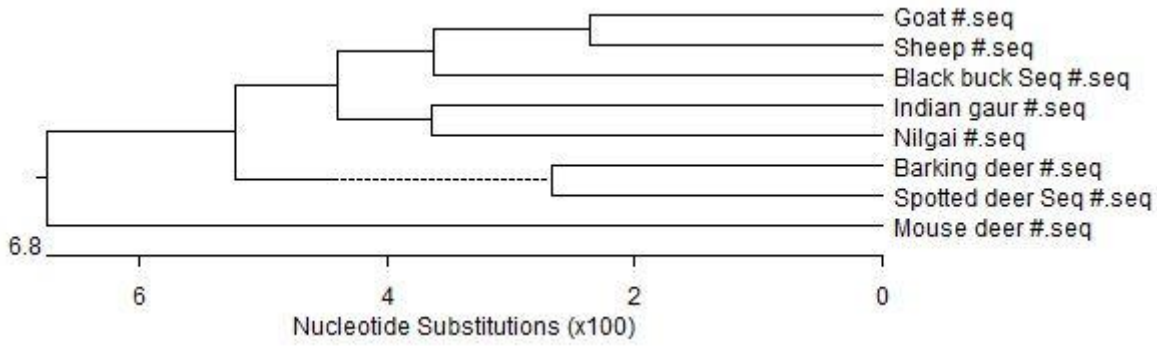

Figure 3: Phylogenetic tree constructed based on the mitochondrial 12S rRNA gene sequences

Wild animal species are threatened by habitat loss, degradation, poaching (Choudhury, 2002), competition from domestic livestock (Pasha *et al*., 2004), disease outbreaks etc. The results of the study indicated the use of universal primers for the amplification of a conserved region of mitochondrial 12S rRNA gene and its sequences analysis using bioinformatics tools could correctly identify species due to multiple polymorphic sites between species (Barbara *et al.*, 2015; Yang *et al.*, 2014; Panday *et al.*, 2014). Based on the precise wild animal species confirmation in the evidence of biological specimen, legal action can be initiated and poaching could be prevented.

Conclusion

Forensically informative nucleotide sequencing is one of the most authentic technique for species identification of biological samples derived from wild ungulates. The technique involves extraction of DNA from the forensic sample, PCR amplification of mitochondrial 12S rRNA gene using the universal primers, sequencing of the amplicon and sequence alignment using the NCBI database. Analysis will give the species to which the sequence matches thereby enables the unambiguous identification of species of meat. Sequence data can be an important evidence for the investigators in the legal process. It can be concluded that, FINS is a robust tool which can be handy to forensic specialists involved in species identification of wild ungulates.

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Conflict of Interests

There is no conflict of interest.

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