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Wildlife Forensics: A Case Study on Golden Jackal *Canis aureus* in India Using Canid Head and Tailor-Made Musk Pod

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The escalating trend of illegal trade on wildlife and wildlife products is a serious threat to existence of wildlife globally. Effective enforcement of laws to contain this requires accurate identification of the seizures which is often difficult due to fake lookalike articles. The articles thus pose challenging complexity and necessitate the usage of different approaches in identifying the species. Species identification by the use of molecular technology is widely recognized to be a definitive method. In this report, we describe the identification of seized canid head, suspected to be of jackal and a pod suspected to be of musk deer using the DNA-based approach. The mitochondrial regions of the query samples were amplified using ribosomal markers, 12S rRNA and 16S rRNA. The sequence reads were compared with the homologous hits extracted from the NCBI database. The phylogenetic analysis was carried out with the aligned sequences to identify species with 500 bootstrap replications. Based on the genetic analysis of both the seizures, the tested samples were identified to be belonging to the species 'Golden Jackal'.

Keywords: Illegal wildlife trade; golden jackal; musk pod; molecular forensics.

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1. INTRODUCTION

Use and abuse of wild animals for the benefit of humankind has been prevalent since ancient times. Apart from live animal trafficking, the illegal trade of wildlife products is contributing to a significant portion of wildlife seizure records (Schefers et al. 2020). Such illicit trade is a complex and lucrative industry worldwide causing threats to both flora and fauna [1]. Though most of the wildlife species are under protection on the national and international scale, only charismatic species like Tiger (*Panthera tigris*) or Asian Elephant (*Elephas maximus*) gains greater concern from the public, the lesser known or less charismatic species do not garner enough attention [2]. Crime on such species generally has a low conviction rate and are under-punished [3].

One such species is Golden Jackal *(Canis aureus)*. They belong to the Canidae family, which is one of the three *Canis* species occurring in India, such as the Indian Wolf *(Canis lupus pallipes)*, the Himalayan Wolf *(Canis himalayensis)* and the Golden Jackal *(Canis aureus)* [4]. This animal with stable population and wide geographic range is protected under Schedule II Part II of the Wildlife (Protection) Act 'WPA' (1972), 'Least Concern' in IUCN red list, and is listed in CITES Appendix III only for India [5]. In spite of their major distribution outside the protected areas, Jackals face drastic decline in their population size due to anthropogenic activities [6]. Such activities cause survival threats to the animal in the form of hunting and poaching for their body parts like skin, teeth, tail, and specifically for jackal 'siyarsinghi' which translates to 'Jackal horns' [3]. It is used in black magic and other superstitious practices in various parts of India [4].

The species identification from the evidences obtained from wildlife crimes usually present a tricky challenge for morphological examinations [7]. Since samples such as dried or processed skin is difficult to analyze, the species identification through DNA-based techniques are more applicable and are well accepted as the foolproof evidence by law [8]. The DNA barcoding technology is being utilized as a potential tool in wildlife forensics, wherein short regions of mitochondrial DNA (mtDNA) are amplified and sequenced to barcode or identify the species with utmost specificity and distinction from other species [9]. Here, we report on wildlife forensic case study undertaken to ascertain species identity by conducting the molecular analysis using 16S and 12S mtDNA markers on excessively dried and processed skin obtained from the seizures of a canid head and a suspected musk pod sample presented by the State Forest department officials for implementation of Wildlife (Protection) Act, 1972 of India.

2. MATERIALS AND METHODS

2.1 Case History

The State Forest department officials confiscated two different seizures in Tamil Nadu. The investigating team had collected the evidences of a canid head and a suspected musk pod from the crime sites. The same was forwarded to the Centre for Wildlife Forensic Sciences, Advanced Institute for Wildlife Conservation Institute (AIWC), Vandalur, Chennai. The cases were registered to undertake molecular analysis for species identification. The canid head sample was marked as 'Seizure 1' and the suspected musk pod was marked as 'Seizure 2' (Fig. 1).

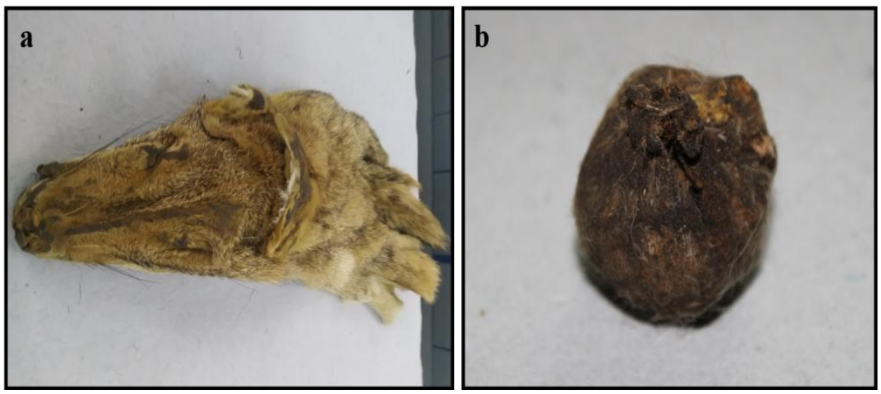

Fig. 1. Photographs of seized a. Canid head (Seizure 1) and b. suspected musk pod (Seizure 2)

2.2 Laboratory Procedures

2.2.1 Washing

One sq.cm of skin sample from Seizures 1 and 2 were taken in separate 2 ml microcentrifuge tubes and subjected to washing with autoclaved distilled water for 5 minutes as samples were excessively dry (seizure 1) and was suspected to have charcoal contamination as filling material in pod (seizure 2). Upon washing, the samples were transferred to fresh tubes for DNA extraction. In case of seizure 2, both interior and exterior skin portions were taken for examination.

2.2.2 DNA extraction, PCR amplification and Sequencing

DNA was extracted following the commercially available Qiagen DNeasy Blood & Tissue kit procedure (Qiagen, Germany) with an extraction blank. The samples were kept under extraction digestion with digestion buffer and proteinase K (KAPA Biosystems, SIGMA), for the period of 48 hours in total, of which 24 hours was in room temperature and 24 hours in circulating water-bath at 56 ℃, in order to achieve utmost digestion of the samples. The DNA extracted from the samples were subjected to amplification of mitochondrial regions of 16S rRNA [10] and 12S rRNA [11] yielding 600bp and 215bp respectively (Supplementary Table 1). Independent PCR for both the target genes was carried out in Eppendorf Nexus GSX1 Mastercycler. The reaction tubes of 10 µL total volume were set up containing 1X Taq Buffer (KAPA Biosystems, SIGMA), 0.25 mM dNTPs, 0.4 µM of both forward and reverse primers, 2.5 mM $MgCl_2$, 0.25 U Taq DNA Polymerase (KAPA Biosystems, SIGMA) and 1 µL of template DNA. Cycling conditions consisted of 5 min of initial denaturation at 95 ˚C, followed by 35 cycles of 30 seconds of denaturation at 95 ˚C, 30 seconds of annealing at 57 ˚C, 45 seconds of extension at 72 ˚C and final extension at 72˚C for 10 min. The PCR reactions were set with positive and non-template controls. Amplicons were visualized in 2% agarose gel with novel juice DNA stain (SIGMA) and documented using BioRad XR+ gel doc system. The PCR products were purified using QIAquick gel extraction kit (Qiagen, Germany) and sequenced bi-directionally by Sanger sequencing in ABI 3730 DNA Analyzer (Applied Biosystems, USA) using ABI Big Dye TM Terminator Cycle sequencing kit (Applied Biosystems, USA). The forward and reverse sequences of each sample were aligned and trimmed at both ends and assembled using MEGA X software.

2.2.3 Data analysis

The sequences of each sample were searched against the GenBank database using BLASTn. The top hits of homologous sequences with a percentage match of greater than 98% were downloaded from GenBank and subjected to multiple sequence alignment with the query sequence using CLUSTALW. Genetic distances/ evolutionary divergence matrix and phylogenetic analysis by Neighbour Joining method using the Kimura-2-Parameter model with 500 bootstrap replications were computed using MEGA X software. The sequences including similar and closely related species with 3 hits for each species were selected with respect to each gene. For 16S rRNA region, the sequences extracted from the NCBI database are of Golden Jackal *(Canis aureus)*, Domestic Dog *(Canis lupus familiaris),* Grey Wolf *(Canis lupus)* and Japanese Wolf *(Canis lupus hodophilax)* for analysis. In the similar fashion, the species picked for 12S rRNA regions are Golden Jackal *(Canis aureus)*, Coyote/ American Jackal *(Canis latrans)*, Grey Wolf *(Canis lupus),* and Red Wolf *(Canis rufus).* The difference in the selected species for 16S rRNA and 12S rRNA is based on the top hits or entries obtained upon search of the query sequences against the NCBI database. For analysis purpose all the query sequences and the extracted sequences from NCBI database were trimmed to same length.

3. RESULTS AND DISCUSSION

Genomic DNA concentration of the isolated samples ranging from 10 - 40 ng/µL was obtained. The extraction control was eliminated from further processing, as no PCR amplification was observed. The mitochondrial genes (16S rRNA and 12S rRNA) from both the seizures were successfully amplified and sequenced. The 12S rRNA assembled sequences of seizure 1 and seizure 2 were 100% similar to the Indian Golden Jackal *(Canis aureus).* Similarly, 16S rRNA sequences of seizure 1 and seizure 2 were 99.54% similar to the Indian Golden Jackal *(Canis aureus).* The query samples of each primer were submitted to NCBI database with the

accession numbers OL960585, OL989144, OL989145 and OL960565. To validate the BLAST search results, evolutionary divergence matrix, neighbour-joining (NJ) tree was constructed for each gene region [12]. In evolutionary distance matrix construction, the number of base substitutions per site between sequences are displayed [13]. The distance computed between the query sequences and the best-hit species from the NCBI database range from 0.000 to 0.013 for 16S rRNA sequences. The least divergence from the query sequences was observed in Domestic Dog *(Canis lupus familiaris)* with 0.010 and most divergence 0.013 was observed with the species Grey Wolf *(Canis lupus)* and Japanese Wolf *(Canis lupus hodophilax)* (Table 1). The distances between 12S rRNA sequences of query samples and NCBI database entries range from 0.000 to 0.005. It is observed from the matrix that query sequences do not have any divergence with closely related species such as Coyote/ American Jackal *(Canis latrans),* and Grey Wolf *(Canis lupus).* The divergence was observed only with Red Wolf *(Canis rufus)* (0.005) (Table 2).

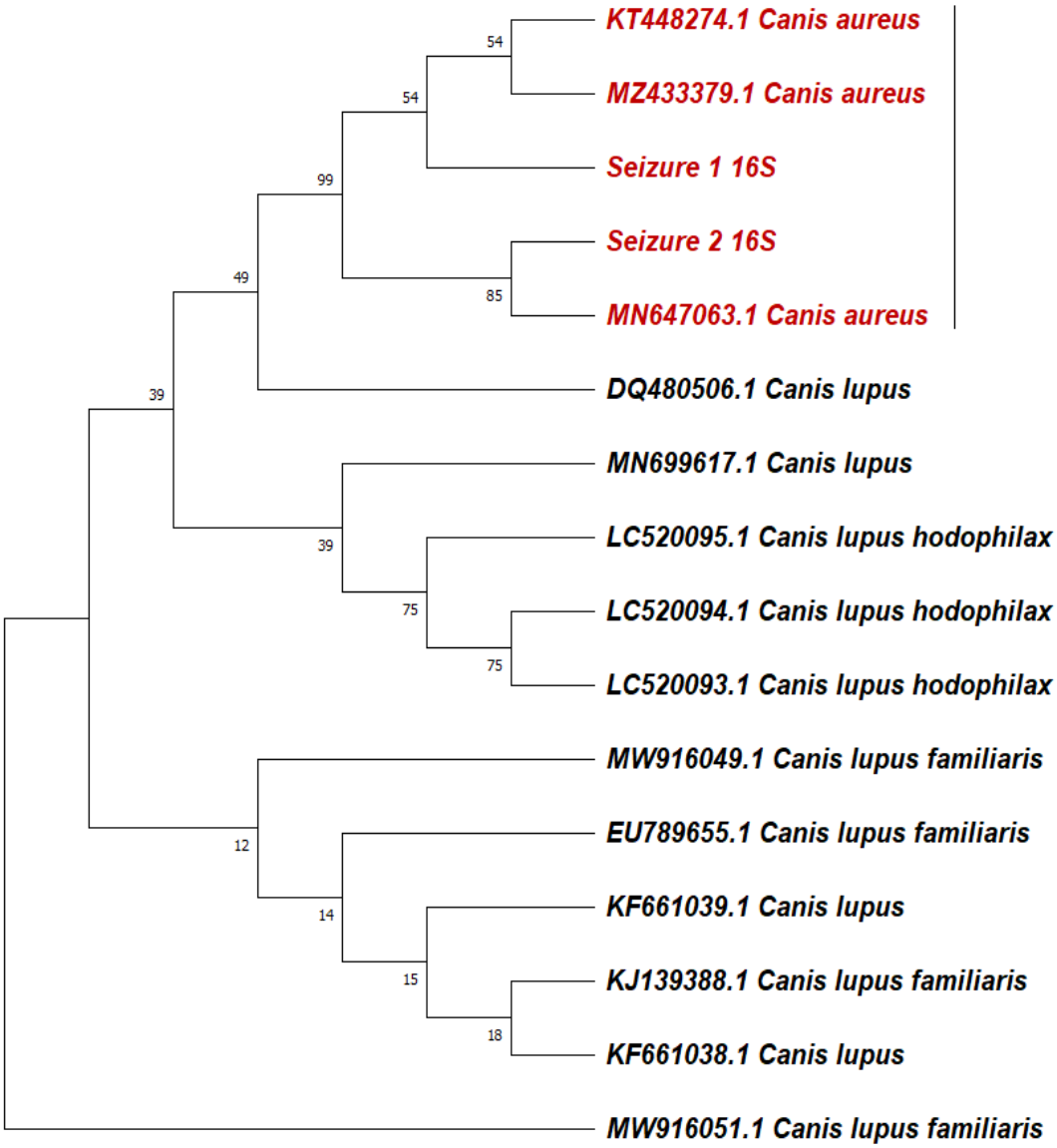

Fig. 2. 16S rRNA gene region-based tree topology displaying the query sequences as Seizure 1 & 2 with the most similar species obtained from the NCBI database through BLAST search

Table 1. Estimates of evolutionary divergence between related species of 16S rRNA gene region. The standard error estimations are shown above the diagonal within the table. The analysis was carried out using Kimura two-parameter model

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Seizure_1_16S		0.000	0.000	0.000	0.000	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
Seizure_2_16S	0.000		0.000	0.000	0.000	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
KT448274.1 *Canis aureus*	0.000	0.000		0.000	0.000	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
MZ433379.1 *Canis aureus*	0.000	0.000	0.000		0.000	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
MN647063.1 *Canis aureus*	0.000	0.000	0.000	0.000		0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
KJ139388.1 *Canis lupus familiaris*	0.011	0.010	0.011	0.011	0.011		0.000	0.000	0.000	0.002	0.003	0.000	0.002	0.002	0.002
MW916051.1 *Canis lupus familiaris*	0.011	0.010	0.011	0.011	0.011	0.000		0.000	0.000	0.002	0.003	0.000	0.002	0.002	0.002
MW916049.1 *Canis lupus familiaris*	0.011	0.010	0.011	0.011	0.011	0.000	0.000		0.000	0.002	0.003	0.000	0.002	0.002	0.002
EU789655.1 *Canis lupus familiaris*	0.011	0.010	0.011	0.011	0.011	0.000	0.000	0.000		0.002	0.003	0.000	0.002	0.002	0.002
MN699617.1 *Canis lupus*	0.013	0.012	0.013	0.013	0.013	0.002	0.002	0.002	0.002		0.002	0.002	0.000	0.000	0.000
DQ480506.1 *Canis lupus*	0.011	0.012	0.011	0.011	0.011	0.004	0.004	0.004	0.004	0.002		0.003	0.002	0.002	0.002
KF661039.1 *Canis lupus*	0.011	0.010	0.011	0.011	0.011	0.000	0.000	0.000	0.000	0.002	0.004		0.002	0.002	0.002
LC520095.1 *Canis lupus hodophilax*	0.013	0.012	0.013	0.013	0.013	0.002	0.002	0.002	0.002	0.000	0.002	0.002		0.000	0.000
LC520094.1 *Canis lupus hodophilax*	0.013	0.012	0.013	0.013	0.013	0.002	0.002	0.002	0.002	0.000	0.002	0.002	0.000		0.000
LC520093.1 *Canis lupus hodophilax*	0.013	0.012	0.013	0.013	0.013	0.002	0.002	0.002	0.002	0.000	0.002	0.002	0.000	0.000	

Table 2. Estimates of evolutionary divergence between related species of 12S rRNA gene region. The standard error estimations are shown above the diagonal within the table. The analysis was carried out using Kimura two-parameter model

Species	1	2	3	4	5	6	7	8	9	10	11	12	13
Seizure_1_12S		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.004	0.005
Seizure_2_12S	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.004	0.005
KT448274.1 *Canis aureus*	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.004	0.005
MZ433379.1 *Canis aureus*	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.004	0.005
KT448277.1 *Canis latrans*	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.005	0.004	0.005
KT448275.1 *Canis latrans*	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.005	0.004	0.005
KT448276.1 *Canis latrans*	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.005	0.004	0.005
MZ042364.1 *Canis lupus*	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.005	0.004	0.005
MZ042363.1 *Canis lupus*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.005	0.004	0.005
MZ042362.1 *Canis lupus*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.005	0.004	0.005
MZ367921.1 *Canis rufus*	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005		0.006	0.006
MZ367912.1 *Canis rufus*	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.009		0.006
MZ367903.1 *Canis rufus*	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.009	0.009	

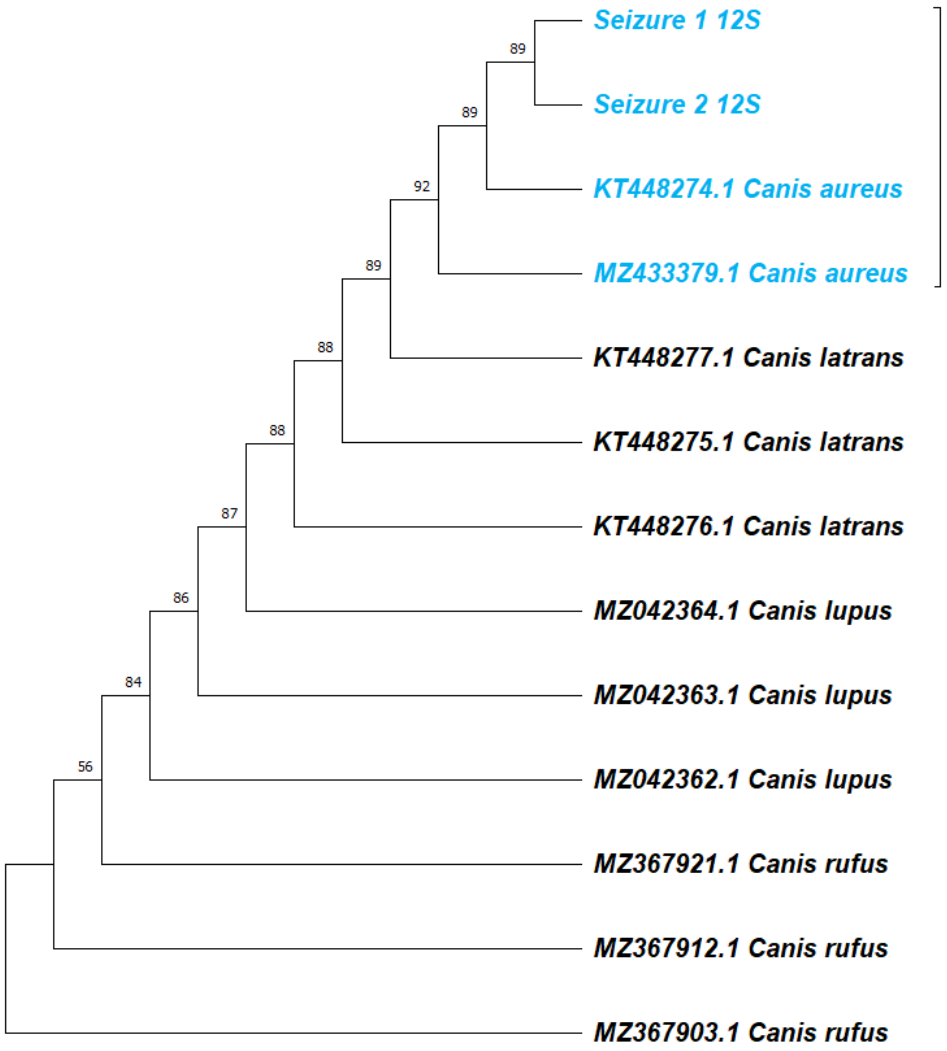

Fig. 3. 12S rRNA gene region-based tree topology displaying the query sequences as Seizure 1 & 2 with the most similar species obtained from the NCBI database through BLAST search

The NJ tree analysis based on 16S ribosomal RNA region of mitochondrial gene showed the query sample of both the seizures 1 & 2 clustering with the *Canis aureus* with 99% bootstrap value and other related species such as *Canis lupus familiaris, Canis lupus, and Canis lupus hodophilax* were grouped separately (Fig. 2). Likewise, 12S ribosomal RNA mitochondrial gene-based tree displayed clustering of query sequences with *Canis aureus* with 89% bootstrap value. The other species included for the analysis grouped one within the other (Fig. 3).

Generally, the primary choice of markers is either cytochrome b or COI for species identification of mammals [14]. Since, the query samples failed in amplification of aforementioned markers, ribosomal markers of mitochondria are used for the study. As far as the employed marker provides a convenient, and accurate species identification, any candidate marker can be used for examination. From the above analysis undertaken on query sequences of mammalian head and suspected musk pod, it is visibly observed that the examination with a single marker is insufficient as closely related species are most likely to produce similar results within a truncated sequence amplification range [15]. In the analysis carried out on 12S rRNA region, it is observed that our query sequences belonging to *Canis aureus* has 100% similarity with other related species of study *Canis latrans* and *Canis lupus* with 0.000 genetic distances. It shows variation only with *Canis rufus* by 0.005

divergence. Though, 16S query sequences displays discrimination with self and related species, the identity confirmation was achieved based on the results of 2 markers. Hence, we advise the use of more than one molecular marker to confirm the species identity, as mentioned in other studies [16].

The present work targeting the Golden Jackal *(Canis aureus)* underscores the need for more attention to control its prohibited trade as it is typically neglected because of its 'common status' and Least Concern IUCN state in India. Illicit trade of such a lesser-charismatic species receives hardly any attention causing their decline or loss [2]. The prevalent paucity of attention on jackal hunting provides favourable ground to the poachers or traffickers for the practice of religious or superstitions beliefs involving wildlife articles such as Jackal horns, skin, and skull. Particularly, in the black-magic world, the demand for the supply of musk-pod pushes the sale of fake pods made of jackal skin [17,18].

4. CONCLUSION

The increasing incidence of wildlife crimes involving not-so attractive wildlife populations pose an unknown threat to the ecosystem globally. Identification of wildlife artefacts by means of physical examination using morphometry usually suffers from lack of reference samples for comparison and also often fails the test of legal scrutiny. DNA examination of wildlife crime samples is considered to be failsafe evidence for species identification. On completion of DNA analysis, we observed that *Canis aureus* is the species of origin for both canid head and the suspected pod, which was confirmed to be a tailor-made pod, reflective of fake pods' circulation in the illegal market. Hence, we recommend the state forest department to conduct capacity-building programs for the forest personnel guarding wildlife, and awareness camps for the public to raise awareness on Golden Jackal and its parts circulating in the illegal wildlife market.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Table 1. List of primers used in the study

mtDNA regions	Primer name	Sequence (5'-3')	Ta (°C)	Amplicon size (bp)
16S rRNA [9]	16L Fwd	CGCCTGTTTATCAAAAACAT	57	600
	16L Rv	CTCCGGTTTGAACTCAGATC		
12S rRNA [10]	12SrRNAL1085 - F	CCCAAACTGGGATTAGATACCC	57	215
	12SrRNAH1259 - R	GTTTGCTGAAGATGGCGGTA		

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