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DIRECT PCR-RFLP BASED SPECIES IDENTIFICATION OF INDIAN WILD CARNIVORES BY NON-INVASIVE SAMPLING

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ABSTRACT

In field conditions such as forest ranges, movement corridors or in areas prone to man animal conflict very often the only evidence/specimens available for investigation are animal scat, fur, hairs, epidermis, etc. Since animal scat contains denuded intestinal epithelial cells it can provide genetic material which can be used for further analysis and other downstream studies. In present study surface scrapings from faecal samples of 5 different carnivore species *viz*., tiger (*Panthera tigris*), lion (*P. leo*), leopard (*P. pardus*), domestic dog (*Canis familiaris*) and domestic cat (*Felis catus*) were collected for DNA extraction using standard kit based protocols. A fragment of *16S rRNA* gene of mitochondrial DNA was amplified using specific primers. The amplicons generated were subjected to restriction digestion. Species identification and differentiation was done using fragment length polymorphism (PCR-RFLP). The results depicted the utility and effectiveness of molecular scatology based PCR-RFLP technique for identification of wild carnivore species in forensic investigations. The observations also highlighted the potential of highly conserved mitochondrial DNA markers like *16SrRNA* gene to be employed for species identification and differentiation of wild carnivores.

Keywords: Carnivores, forensic, markers, mtDNA, PCR-RFLP, scatology, speciation, sympatric

INTRODUCTION

Worldwide there are 36 recognized species of wild cats, of which 15 species are found in India which include 5 species of large cats *viz*., tiger (*Panthera tigris*), lion (*P. leo*), leopard (*P. pardus*), snow leopard (*Uncia uncial*) and clouded leopard (*Neofelis nebulosa*)]. Genetic studies using non-invasive samples like scat or fur are of paramount importance in ecological and behavioural studies involving wild carnivores which aid wildlife biologists in habitat management and other conservational measures directed at threatened/ endangered wild carnivores (Laguardia *et al.*, 2015). Species identification and differentiation is essential for devising mitigation strategies aimed at minimizing the man-animal conflicts and other forensic investigations (Reed *et al.*, 1997). Animal scat provides sufficient genetic materials which can be analyzed for monitoring wild carnivores in their habitats, tracking their movement and in delineation of core areas and buffer zones (Lucchini *et al.*, 2002).

The traditional methods of tracking and monitoring wild carnivores include capture methods, camera trapping, telemetry, radio collaring or blood/tissue collection which are costly and labour intensive; and pose risk of injury to both animal and handler (Paxinos *et al.*, 1997). The distribution

studies of wild carnivores have been conducted on the basis of their scat morphologies (Mukherjee *et al.,* 2010). This method can often be misleading; however, non-invasive method using DNA from faecal samples is a safe alternative approach to study the ecology and distribution of wild carnivores. Scat samples of animals can be identified and categorized by species, individual, gender and even population dynamics if a large number of samples are collected for the analysis of DNA extracted from them (Kitano *et al.,* 2007). For DNA extraction faecal samples are superior over other non-invasive field samples like hair or fur as it provides sufficient genetic material to avoid problems of allelic dropout or ghost alleles while genotyping. Animal scat contains sloughed epithelial cells of intestinal lining and provides sufficient genetic information which could be used to identify species on a wide variety of criteria with proper training and analysis (Wasser *et al.,* 1997). Molecular scatology is particularly important in monitoring elusive and rare carnivores for which faecal materials are often the only sample available for analysis (Hoss *et al*., 1992; Palomeres *et al.,* 2006). The success and error rate of faecal DNA based studies depends on a variety of factors especially season of collection, diet of animal, age of scat, collection and storage method employed (Lucchini *et al.,* 2002). Similarly, the area of faecal deposit to be used for sample collection is also very important, surface scrapings or samples collected from outer surface of faecal deposit depicted higher amplification success rate as compared to those collected from interior of deposit or homogenized scat samples (Flagshed *et. al.,* 1999). There are higher chances of contamination of samples during collection process, therefore the instruments should be sterilized and scat samples be placed individually in sterile zip-lock bags or containers and transferred immediately to -20ºC for storage until further processing (Goosens and Milena, 2013). However, the analysis of DNA from faecal samples can be challenging because faecal DNA extracts often contain high concentration of PCR inhibitors (Wasser *et al*., 1997; Paxinos, 1997; Cossios and Beruard, 2006). Therefore, it is necessary to design an extraction method which will minimize inhibitors while maximizing DNA yields. Although there is no clear consensus regarding the best method of faecal DNA extraction as it may vary within species or geographical area but currently the silica binding extraction kit method is most commonly used method that has proven effective to a greater extent (Reed *et al*., 1997). A comparative study on different types of faecal DNA extraction methods including proteinase K, guanidine thiocyanate, QIAgen QIAamp stool kit and modified QIAgen QIAamp stool kit method revealed that QIAgen QIAamp stool kit method yielded best results in terms of DNA quantity and quality (MCleeve, 1998).

In samples containing a very small amount of DNA such as faeces and hair/fur materials, mitochondrial DNA might be the only source of analysis because of more number of copies per cell than nuclear DNA (Branicki *et al*., 2003). On the basis of analysis by various techniques, mitochondrial markers including *cytochrome b*, *cytochrome oxidase (I-III), 12S rRNA* and *16SrRNA* genes have been found better suited for species identification because of the presence of some highly conserved regions that show minimal intra-species and maximum inter-species variations in nucleotide sequences (Rastogi *et al*., 2007). On the other hand, nuclear DNA is well-suited for individual or gender discrimination. The species identification can be done using DNA extracted from faeces either by detection of species-specific nucleotide sequence patterns and subsequent phylogenetic analysis or by using direct methods like PCR-RFLP and single strand conformation polymorphism (Palomeres *et al*., 2002), however direct methods are cost-effective and require lesser time period. PCR-RFLP technique using mitochondrial DNA has successfully been employed to differentiate otters, American mink, pole cats in Europe and most carnivore species in north America (Mills, 2000). Andean felids have also been successfully differentiated at species level by using PCR-RFLP technique following the amplification of 16S *rRNA* gene of mitochondrial DNA (Cossios and Bernard, 2006). Evidences show that the direct methods which need no prior sequencing information such as RAPD, AFLP and RFLP can be used effectively for species identification of animal components containing even less amount or low quality DNA e.g. scats, hair/furs, carcasses as well as museum skins or bones (Mukherjee *et al.,* 2010). However, the problems are interpretation of results in case of complex mixtures where 2 or more species are to be detected (Bottero *et al.,* 2003). Further requirement of large fragment size is also likely to limit the application of these techniques. In present study faecal DNA extracts from 5

animal species leopard (*Panthera pardus*, Pocock, 1917), Asiatic lion (*Panthera leo*, Pocock, 1917), tiger (*Panthera tigris*, Pocock, 1917), domestic cat (*Felis catus*, Linnaeus, 1758) and domestic dog (*Canis familiaris*, Linnaeus, 1758) were used for molecular scatology analysis. The study was conducted in two stages, in stage I following the amplification of target *16S rRNA* gene segment, restriction digestion and species discrimination by fragment length polymorphism was done for leopard, domestic cat and domestic dog. In stage II, the amplicons of *16S rRNA* gene segment of 5 carnivore species were subjected to restriction enzyme digestion; and species identification was done on the basis of polymorphism of digested fragments. Faecal samples of domestic dog and cat were included in the study because in areas of man-animal conflict especially areas in the vicinity of forests, the faecal deposits of these animals are frequently encountered due to their abundance near human habitations and it may create confusions in forensic studies. The present study was aimed to assess the sustainability and effectiveness of animal scat based PCR-RFLP technique to address scat identification dilemma of Indian carnivores especially in situations where faecal samples of 2 or more animal species are collected from field for forensic analysis.

MATERIALS AND METHODS

Scat samples were collected from leopards (*Panthera pardus*) (N = 8), tigers (*P. tigris*) (N = 8) and Asiatic lions (*P. leo*) (N = 8) at Arignar Anna Zoological Park (AAZP) Vandalur, Chennai (India). Also, faecal samples were collected from domestic dogs (n = 15) and cats (n = 15) from clinical block and inpatient ward of Madras Veterinary College, Chennai (India). Surface scrapings of scat samples were taken using sterile surgical blades (BP blades) in labelled sterile plastic containers and immediately transferred to laboratory in ice flask for storage at -20ºC until further processing.

DNA extraction protocol

From each sample, 200 mg scat was transferred to labelled 2 mL micro-centrifuge tubes and 1 mL inhibitex buffer added to each tube followed by vortexing until the sample was thoroughly homogenized. The samples were centrifuged for 1 min to pellet scat particles. Following this new 1.5 mL labeled centrifuge tubes were taken and 25 µL proteinase k was added to each tube. Then, 600 µL supernatant was pipetted out of 2 mL tubes and was transferred into new 1.5 mL tubes containing proteinase K. Then, 600 µL buffer AL was added to each tube followed by vortexing for 15 sec; and incubated at 70ºC for 40 min. After incubation, 600 µL ethanol (96-100%) was added to lysate, mixed by vortexing; and 600 µL lysate from each tube was transferred into labelled QIAamp mini spin columns followed by centrifugation at 14000 rpm for 1 min. The spin columns were then placed in new 2 mL collection tubes and the previous tubes were discarded. Again, 600 µL lysate was charged on each column from respective micro-centrifuge tubes followed by centrifugation at 14000 rpm for 1 min. The procedure was repeated until all the lysate from tubes was transferred to spin columns. The QIAamp mini spin columns were then transferred into new collection tubes and 500 µL buffer AW1 was added to each spin column and were then centrifuged for 1 min at 14000 rpm. The spin columns were again placed in new collection tubes and 500 µL AW2 was added to each tube followed by centrifugation at 14000 rpm for 3 min. Finally, the spin columns were placed in new labeled 1.5 mL micro-centrifuge tubes and 100 µL buffer ATE was directly added to membrane of each spin column and were incubated for 10 min at room temperature. The tubes containing spin columns were centrifuged at 14000 rpm for 2 min to elute the DNA and the extracted DNA was stored at -20ºC until further processing.

Determination of yield and purity of DNA

The yield of DNA from faecal samples was estimated by the use of Nano-drop technology in terms of ng uL^{-1} and the purity of DNA was determined as $0D_{260}$:OD_{280} ratio. All the samples were analyzed with Nano-drop and observations recorded precisely. The extracted faecal DNA was further subject to

0.8% agarose gel electrophoresis at 70V, 500 mA current for a period of 50 min and bands generated were observed in a GelDoc (Bio-Rad, USA) system for checking quality and quantity of DNA.

Amplification of target gene sequence

The full nucleotide sequences of *16S rRNA* gene were obtained from NCBI for the five animal species included in the study in FASTA format. The amplification of *16S rRNA* gene was carried out by using universal forward and reverse primers developed by Mukherjee *et al*. (2010) (forward: GCCTGTTTACCAAAAACATCAC, and reverse: CCTAGGGTAACTTGTTCCGTTG). To avoid mispriming of the selected primers with any other gene in target species, the designed primer sequences were blasted to non-redundant (nr) database of NCBI to determine similar sequences through nblast (*www.ncbi.nlm.nih.gov*). The blasted primers should have lesser 'E' values with the target gene. 'E' value or expected value is the measure of the reliability of 'S' score. It is the parameter that describes the number of hits one can 'expect' to see by chance when searching a database of particular size. It decreases exponentially as the score (S) of the match increases. Since 'E' value of selected primer sequences was least for the target genes than any other gene, hence the chosen primers were considered best for the amplification of target gene. The primer sets were diluted as per the instructions in primer synthesis report to obtain a stock primer solution having a final concentration of 100 pmol μL^{-1}. Then the primers were further diluted (1 in 10 dilution) to a working solution of 10 pmol μL^{-1} concentration with millipore water (45 µL millipore water + 5 µL primer stock solution). The annealing temperature of each primer set (forward and reverse primers) was determined by carrying out gradient PCR, based on the melting temperature of primer set. The temperature which provided a good and specific PCR product yield was chosen to be the annealing temperature of primer set. A 20 µL PCR mixture was prepared comprising of following ingredients; PCR assay buffer (10X) – 2 µL; $MgCl_2$ (1.5-3.0 mM) – 1.8 µL; dNTPs (each 200 µM) - 1 µL; Primers: forward (10 pmoles) reverse (10 pmoles) - 1 µL; Taq DNA polymerase (1 unit) – 0.2 µL; template DNA - 4 Ml and nuclease free water - 10 µL. The temperature-time protocol employed for PCR reaction was: initial denaturation at 95ºC for 5 min, followed by denaturation at 95ºC for 45 sec, annealing at 56ºC for 40 sec and extension at 72ºC for 40 sec. After completing 35 cycles of above mentioned protocol final extension was done at 72ºC for 5 min followed by holding of samples at 4ºC until further processing.

The PCR products were checked on 2% agarose gel. The electrophoresis was carried out at 70 V and 500 mA current for 50 min. The DNA bands were observed in a GelDoc system (Bio-Rad, USA) and upon confirmation of the size of amplified products, six samples from each of the 5 animal species were used to perform amplification of 16S *rRNA* gene.

Restriction enzyme digestion

A web-based NEB cutter V.2 program was used to select restriction enzyme that would differentially cut the *16SrRNA* gene segment for each animal species, and based on this analysis *Taq 1* enzyme was selected. First restriction enzyme digestion was carried for 442 bp size amplicons of leopard, domestic dog and domestic cat with 15 µL reaction volume containing 1 µL enzyme (20 U), 3 µL PCR product and 2.2 µL Cut Smart buffer, and rest volume was made by nuclease free water (NWF). The mixture was incubated at 65ºC for 60 min, followed by enzyme deactivation at 80ºC for 10 min. The fragments generated after incubation were run on 3% agarose gel, electrophoresis was carried out at 90 V and 500 mA current for 60 min. The bands developed were observed in a GelDoc system (Bio-Rad, USA). The second restriction enzyme digestion reaction for the PCR amplicons of partial *16S rRNA* gene sequence for leopard, cat, dog, Asiatic lion and tiger was carried out using the same size (442 bp) amplicons. The final reaction volume (20 µL) was made for each sample using 5 µL PCR product, 1 µL (20 units) enzyme and 2 µL Cut-smart buffer, and the rest volume made up by adding NFW. The material was incubated at 65ºC for 40 min, followed by enzyme deactivation at 80ºC for 10 min. The fragments generated after digestion were run on 3% agarose gel, electrophoresis was carried out at an voltage of 90 V and 500 mA current for 60 min and the products were visualized in GelDoc system (Bio-Rad, USA).

RESULTS AND DISCUSSION

The mean yield and purity of DNA was determined by Nano-drop analysis. The isolated DNA had mean concentrations of 70.76 ± 0. 25, 68.3 ± 0.29, 72.35 ± 0.23, 77.57 ± 0.34 and 75.03 ± 0 31 ng µL for leopard, tiger, lion, cat and dog, respectively. The mean values of OD_{260}:OD_{280} ratios were 1.77 ± 0.59,1.78 ± 0.66 , 1.89 ± 0.67, 1.74 ± 0.61 and 1.84 ± 0.70 for leopard, tiger, lion, cat and dog, respectively. The subjective assessment of quality of isolated DNA through agarose gel electrophoresis revealed a single band without shearing.

The amplification of 16S *rRNA* gene successfully produced 442 bp amplicons for all the 5 species studied (Plate 1). In the first reaction, the restriction enzyme digestion with *Taq I* restriction enzyme produced fragment patterns showing polymorphism among the three species leopard, domestic cat and domestic dog (Plate 2). The leopard 16S*rRNA* gene sequence has two restriction sites for *Taq I* enzyme, generating 3 fragments of 316, 70 and 56 bp length; while the 16S *rRNA* gene sequence of cat had only one restriction site for *Taq I* enzyme yielding only two fragments 387 and 55 bp length

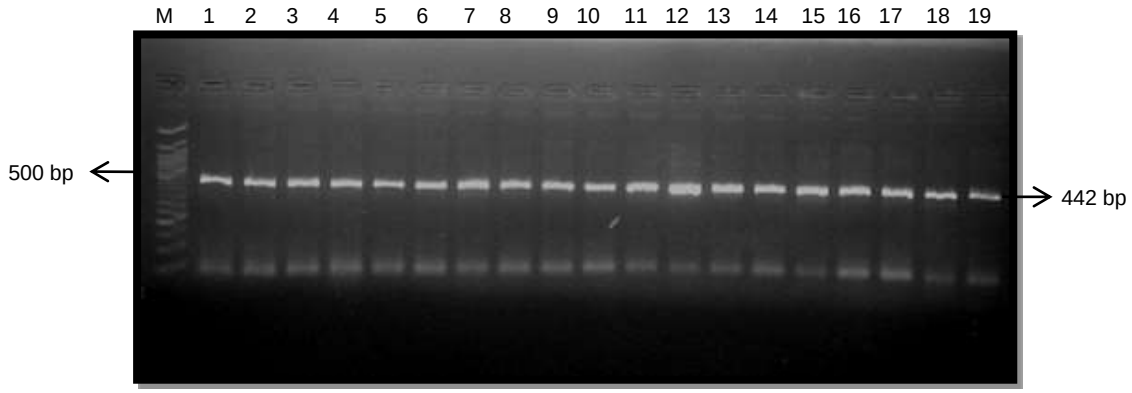

Plate 1: Agarose gel electrophoresis showing PCR products of 16S *rRNA* gene; Lane 1: 50 bp DNA ladder marker; lanes 2-8: 16S *rRNA* gene of leopard; lanes 9-14: 16S *rRNA* gene of cat; and lanes 15-19: 16S *rRNA* gene of dog

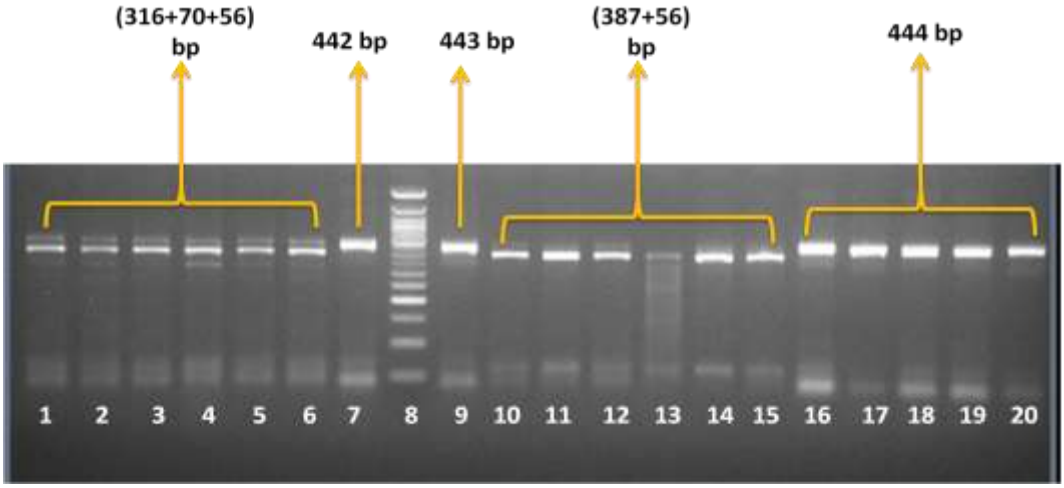

Plate 2: PCR-RFLP fragments of 16S *rRNA* gene of leopard, cat and dog; Lane 1-6: PCR-RFLP of 16S *rRNA* gene of leopard showing the bands of 316, 70 and 56 bp; Lane 7: Undigested 16S *rRNA* gene of leopard showing the band of 442bp; lane-8: 50 bp DNA ladder; lane-9: Undigested 16S *rRNA* gene of domestic cat showing the band of 443bp; Lane 10-15: PCR-RFLP of 16S *rRNA* gene of domestic cat showing the bands of 387 and 56 bp; and Lane 16-20: PCR-RFLP of 16S *rRNA* gene of domestic dog showing the undigested band of 444 bp.

after digestion. On the other hand, the 16S *rRNA* gene sequence of dog did not show any restriction site for *Taq I* enzyme; therefore, the PCR products remained undigested and did not yield any additional fragments after incubation. The occurrence of same fragment patterns in 16S *rRNA* gene sequence of leopard, domestic dog and cat after restriction digestion with *Taq I* enzyme have previously been reported (Mukherjee *et al.*, 2010). Further, the fragment patterns yielded were similar to the expected patterns given by NCBI's NEB cutter V.2 web based program after analyzing the reference sequences of 16S *rRNA* gene derived from Genbank.

In second reaction, the 442 bp partial sequence of 16S *rRNA* gene of five carnivore species i.e. leopard, domestic cat, domestic dog, tiger and Asiatic lion were subject to restriction digestion with *Taq I* enzyme which produced fragment patterns showing polymorphism among the 5 carnivore species. The leopard 16S *rRNA* gene sequence had 2 restriction sites for *Taq I* enzyme, and generated 3 fragments of 316, 70 and 56 bp length; the 16S *rRNA* gene sequence of cat had only one restriction site for *Taq I* enzyme yielding only 2 fragments of 387 and 55 bp length after digestion; tiger 16S *rRNA* segment had 2 restriction sites for *Taq I* enzyme which produced 3 fragments of DNA of 255, 128 and 59 bp length and the16S *rRNA* segment of lion produced 3 fragments of DNA of 312, 71 and 60 bp length depicting the presence of 2 restriction sites for *Taq I* enzyme. On the other hand, the 16S *rRNA* gene sequence of dog does not show any restriction site for *Taq I* enzyme, therefore the PCR products remained undigested and did not yield any additional fragments after incubation. All the five carnivore species were efficiently differentiated from each other by polymorphism shown by the fragment patterns generated after restriction digestion (Plate 3). The restriction enzyme digestion produced fragment patterns that were similar to the expected patterns given by NCBI's NEB cutter V.2 web based program after analyzing the reference sequences of 16S *rRNA* gene derived from Genbank.

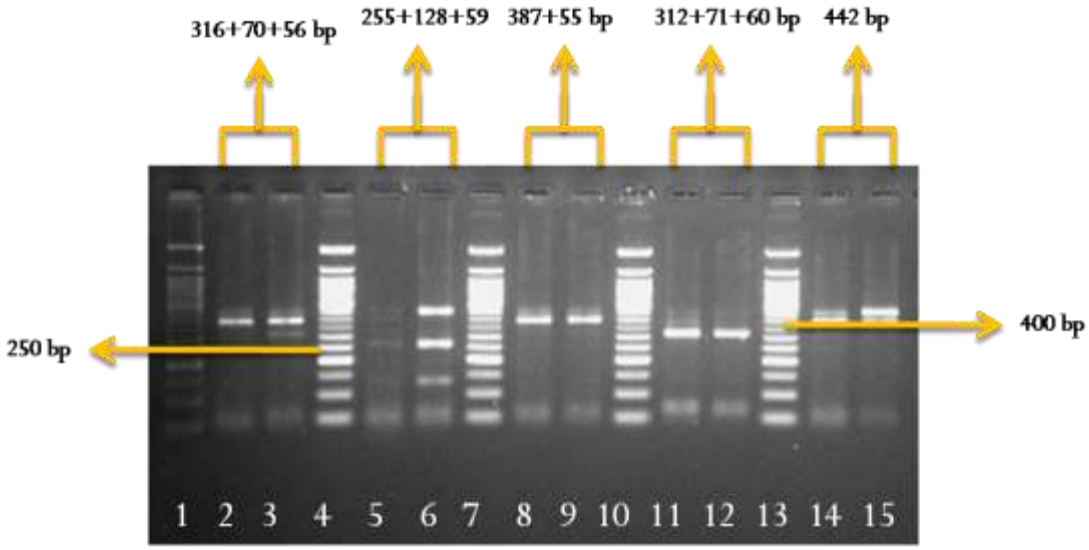

Plate 3: PCR-RFLP fragments of 16S *rRNA* gene of leopard, tiger, cat, lion and dog; Lane 1, 4, 7, 10 and 13: 50 bp DNA ladder; Lane 2 and 3: PCR-RFLP of 16S *rRNA* gene of leopard showing bands of 316, 70 and 56 bp; Lane 5 and 6: PCR-RFLP of 16S *rRNA* gene of tiger showing bands of 255, 128 and 59 bp; Lane 8 and 9: PCR-RFLP of 16S *rRNA* gene of domestic cat showing bands of 387 and 56 bp; Lane 11 and 12: PCR-RFLP of 16S *rRNA* gene of lion showing bands of 312, 71 and 60 bp; and Lane 14 and 15: undigested PCR product band of 442 bp length

The results of enzyme digestion produced fragment patterns that were similar to the expected patterns given by NCBI's NEB cutter V.2 web based program after analyzing the reference sequences of 16S *rRNA* gene derived from Genbank. The polymorphism occurring between the species may be attributed to the variations in nucleotide sequences of 16S *rRNA* gene among these animal species. The position and the number of restriction sites for *Taq* I enzyme within *16S rRNA* gene was different

for these animal species resulting in the generation of different number of fragments of varying size. These findings are in agreement with Mukherjee *et al.* (2010) who reported the occurrence of same fragment patterns in *16S rRNA* gene sequence of leopard, domestic dog and cat after restriction digestion with *Taq* I enzyme. The PCR-RFLP method used in this study is a direct and efficient method which proved very powerful tool for species identification using genomic or mitochondrial DNA extracted from biological materials like faeces, hair, bones, hides, etc. This method is a very good alternative to PCR-sequencing methods for species identification especially under those circumstances where sequencing of DNA is not possible or otherwise this method can be used to augment the findings of sequencing methods as done in this study. This can be a vital tool for differentiation of sympatric carnivores that have overlapping habitats, feeding behaviours and prey preferences making it virtually impossible to identify the species by the appearance of scat mass. However, difficulties may arise in this method while differentiating two closely related species that can potentially hybridize resulting in similar kind of fragment patterns after restriction digestion without considerable polymorphism. In that condition, the use of an additional restriction enzyme would prove/differentiate the closely related species.

Conclusion: The PCR-RFLP method used is a direct and efficient method and proved a very powerful tool in species identification using the DNA extracted from biological materials. This method is an alternative to PCR-sequencing methods for species identification especially under the circumstances when DNA sequencing is impossible. However, difficulties may arise in this method in differentiating two closely related species that can potentially hybridize, resulting in similar kind of fragment patterns after restriction digestion without considerable polymorphism. Under this condition, the use of additional restriction enzyme can be experimented to differentiate the closely related species. The study proved the usefulness of PCR-RFLP technique for differentiating multiple animal species using mitochondrial DNA extracted from faecal samples. This can be a vital tool for differentiation of sympatric carnivores that have overlapping habitats, feeding behaviours and prey preferences which are otherwise difficult to identify by appearance of their scat mass under field conditions.

Ethical statement: The present work was approved by the Ethical Committee of Madras Veterinary College, Chennai (India); and the support provided by the authorities at Arignar Anna Zoological Park (AAZP) Vandalur, Chennai (India) is duly acknowledged.

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