GENETIC CHARACTERIZATION OF CERTAIN CAPTIVE WILD CARNIVORES USING RANDOM-AMPLIFIED POLYMORPHIC DNA-POLYMERASE CHAIN REACTION

B. Nishanth*[1], C. Sreekumar[2], K.G. Tirumurugaan[3], G. Sarathchandra[4] and K. Senthil Kumar[5]

Department of Wildlife Science
Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University
Chennai – 600 007, Tamil Nadu

ABSTRACT

Wildlife species identification, based on DNA is a highly reliable method for the investigation of wildlife crimes. PCR techniques and microsatellite markers are extensively used for the identification of wild animal DNA in forensic cases. Mostly, genes like Cyt B, Cox 1 and 12S RNA are amplified coupled with sequencing, to confirm the species in question. In the present study, random amplified polymorphic DNA (RAPD) PCR was performed with DNA samples from captive wild felids, canids and ursids and the fingerprint pattern was analyzed for their utility in designing de novo diagnostic primers. DNA was extracted from the tissue samples from eleven animals (two numbers each from tigers and lions and one sample each from a leopard, jaguar, sloth bear, black bear, wolf, jackal and dhole). RAPD-PCR was carried out using different arbitrary decamers. Of the five decamers used, two decamers, namely AP7 and AP17 revealed consistent amplification patterns. Among them, AP7 produced monomorphic fragments corresponding to 500 bp for felids and 800 bp and 400 bp for ursids, while polymorphic bands were generated across the different genera. The decamer AP17 consistently amplified a 450 bp band from

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1 Assistant Professor, Directorate of Research, TANUVAS, Chennai-600 051, Tamil Nadu, India. Corresponding author Email id : nishvet@gmail.com
2. Professor and Head, Dept. of Wildlife Science, MVC, Chennai-600 007
3.Project Director i/c., Translational Research Platform for Veterinary Biologicals, TANUVAS, Madhavaram Milk, Colony, Chennai-600 051
4.Professor and Head, Pharmacovigilance Laboratory for Animal Feed and Food Safety, TANUVAS, Madhavaram, Milk Colony, Chennai-600 051
5.Assistant Professor, Post Graduate Research Institute in Animal Sciences, TANUVAS, Kattupakkam, Kancheepuram district – 603 203

all the felid samples and polymorphic amplification with other genera. The potential for these RAPD amplicons for their applicability in the design-specific primers for genus/ species-specific detection of wild animal DNA due to their uniqueness is discussed.

Keywords: Random amplified polymorphic DNA, Fingerprinting, Captive wild carnivores

INTRODUCTION

In the present scenario, wild animals face the risk of population decline, especially due to crimes including poaching and illegal trade. In most cases, the crimes are not revealed immediately. By the time a crime is revealed, the evidence gets destroyed completely or partially. Processed wild animal parts are sometimes very difficult to identify based on morphology alone. Crime scene investigation is done based on the available evidence to identify the animal species involved. Deoxyribonucleic acid (DNA) based modern techniques are popularly used for the identification of species in forensic cases. Various tools like Southern hybridization-based minisatellite analysis (Jeffreys *et al*., 1985; Singh, 1991) and polymerase chain reaction (PCR) based microsatellite genotyping (Ewart *et al*., 2018; Li *et al.,* 2019) were used for DNA identification. The use of universal primers (Verma and Singh, 2003; Naidu *et al.*, 2012) targeting the Cytochrome b region is widely practised for DNA amplification in forensic investigations for species identification. However, the sequence information of the amplified regions is essential for further analysis and comparison based on the reference data in Gen Bank and interpretation of Basic Local Alignment Search Tool (BLAST) search results.

The above methods are used for the analysis of unknown samples and to establish the species of origin. Random amplified polymorphic DNA (RAPD) is a type of PCR where segments of DNA are amplified (Williams *et al.,* 1990) using random primers and the genome can be scanned more randomly than using conventional techniques to produce a fingerprint (Ramella *et al*., 2005). Prior knowledge of the DNA sequence for the target gene is not necessary for this technique, as it involves the amplification of random segments of genomic DNA using a single short primer with arbitrary sequences (Chelomina *et al.*, 1999). Hence, one can expect to generate a fingerprint with similarities/differences in the DNA sequences across different species. In this study, samples belonging to captive wild felids, canids, and ursids were analysed using arbitrary primers (decamers) as molecular markers for generating fingerprint pattern. The applicability of such markers to potentially differentiate different genera and species is discussed.

MATERIALS AND METHODS

Samples

Tissue samples (liver, spleen and brain) of eleven carnivores (two numbers each from tigers and lions and one sample each from leopard, jaguar, sloth bear, black

bear, wolf, jackal and dhole) were collected during the post mortems conducted in Arignar Anna Zoological Park, Vandalur, Chennai, in the year 2020 and 2021. The samples were preserved in 90% alcohol and stored in a deep freezer (-20^{o}C) before DNA extraction.

Extraction of DNA

The tissue samples were subjected to DNA extraction using DNeasy Blood and Tissue Kit following the procedure recommended by the manufacturer. The concentration of the eluted samples was evaluated and stored at a concentration of 20 ng/µl at -20°C for further analysis.

RAPD-PCR

Five random decamers (Table 1, Sreekumar *et al*., 2003) were used to amplify the DNA. Amplification was carried out in a 25 µL reaction volume containing 20 ng of template DNA, 100 µM each of dNTPs, 10 pmole of primer, 1.5 mM $MgCl_2$, and 0.5 units of Ampliqon (Cat. No. A180301) and 1x PCR buffer (10 mM Tris-HCl, ph 8.3 and 50 mM KCl). PCR cycling was performed in a thermal cycler (T100 thermal cycler, Bio-Rad) with a ramp rate of 4°C/sec. The PCR conditions were: initial denaturation at 95°C for 5 min, followed by 45 cycles each of denaturation at 94°C for 1 min, annealing at 36°C for 45 sec, extension at 72°C for 1 min and a final extension at 72°C for 5 min. The PCR assay was repeated five times for reproducibility with nuclease-free water as the negative control. The PCR products were subjected to electrophoresis (80V) in a 2% agarose gel stained with ethidium bromide and the fingerprint patterns were analyzed based on the size.

RESULTS AND DISCUSSION

Among the five decamers used, only two (AP7 and AP17) produced consistent DNA fingerprint patterns in all the samples. Many monomorphic and polymorphic fragments were amplified with the primers AP7 and AP17. The primer AP7 resulted in a specific amplification of a 500 bp fragment (Fig.1) with significant intensity from all feline samples. In the bear samples, two amplicons of 400 bp and 800 bp were observed and they seemed to be unique to the bear species. In the canid samples, the AP7 primer revealed polymorphic fragments ranging from 450 bp to 1000 bp. The DNA from the wolf resulted in a specific amplicon of 600 bp band while a 500 bp fragment was seen for the jackal. In contrast, the dhole samples generated three unique amplicons of sizes 450 bp, 500 bp and 1000 bp (Fig. 1).

The fingerprint pattern observed with the AP17 primer is depicted in figure 2. The primer amplified numerous fragments in the felid samples ranging from 450 bp to 850 bp (Fig. 2), with a specific amplicon of 450 bp amplified with greater intensity than the other fragments. In sloth bear, the AP17 primer revealed polymorphic fragments of 350 bp and a more intense 400 bp product. With the DNA from black, three amplicons of 350 bp, 400 bp, and 450 bp were observed while in the DNA of the wolf three amplicons of sizes 250 bp, 400 bp and 500 bp were observed. When

Table 1: Primers used for RAPD PCR of captive wild felids

Primer	Primer Sequence	Reference
AP7	GTGATCGCAG	Sreekumar *et al.* (2003)
AP10	CCGGTGTGGG	
AP15	CGGACGTCGC	
AP17	TCACGATGCA	
AP22	CTGAGACGGA	

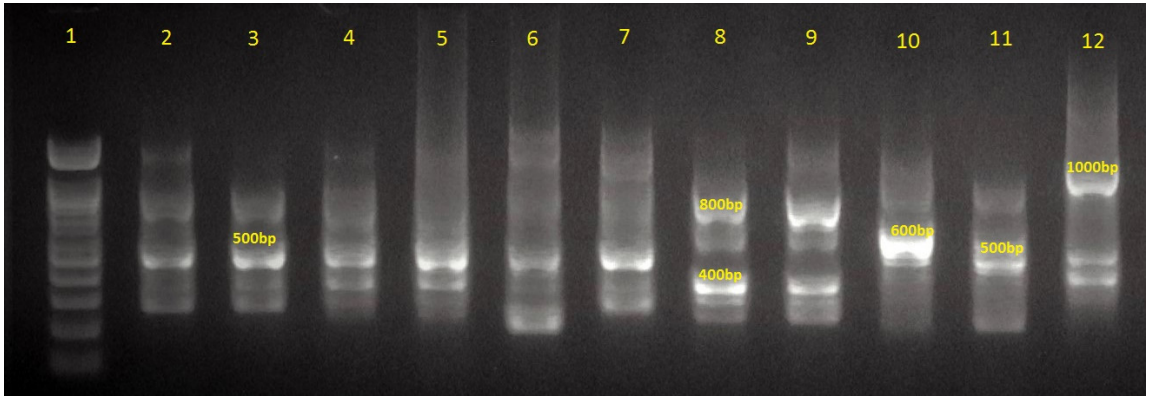

Figure 1: RAPD-PCR fingerprints of felids, ursids and canids with AP7 primer 1-100 bp ladder, 2 and 3-Tiger, 4 and 5-Lion, 6-Leopard, 7- Jaguar, 8-Sloth bear, 9-Black bear, 10-Wolf, 11-Jackal, 12-Dhole

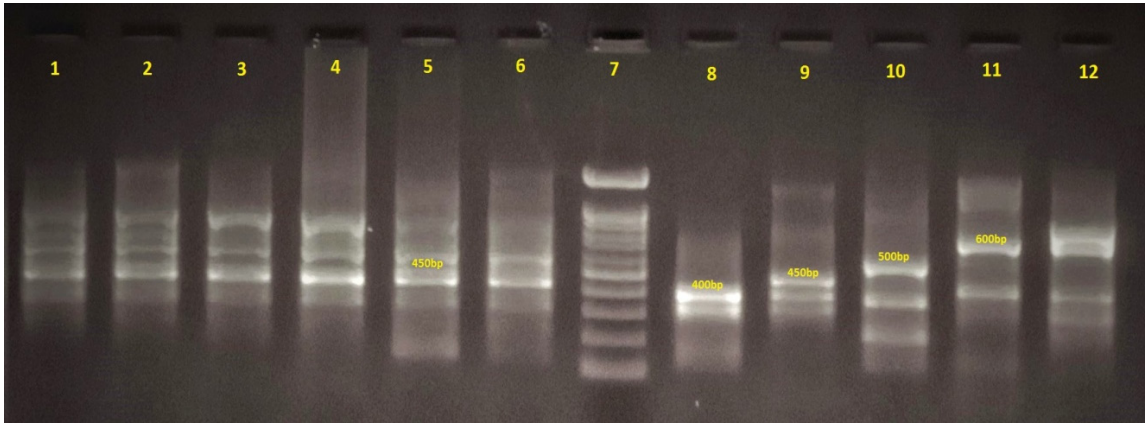

Figure 2: RAPD-PCR fingerprints of felids, ursids and canids with AP17 primer 1 and 2-Tiger, 3 and 4-Lion, 5-Leopard, 6- Jaguar, 7-100 bp ladder, 8-Sloth bear, 9-Black bear, 10-Wolf, 11-Jackal, 12-Dhole

tested with the DNA of jackals, two amplicons of 420 bp and 600 bp were observed. The dhole DNA resulted in four amplicons of about 300 bp, 400 bp, 600 bp and 620 bp repeatedly. The fragments corresponding to 600 bp and 620 bp were brighter than the other two fragments. The other decamers (AP10, AP15 and AP22) did not result in any specific amplicon size with the DNA from the species tested.

Universal primers amplifying the cytochrome Bregion of the DNA are used for forensic investigations in identifying wild animals. This gene has been chosen because this region can be targeted even in DNA extracted from the most degraded parts or tissues, as a small fragment can be easily amplified. The studies carried out by Verma and Singh (2003), Naidu *et al*. (2012), Branicki *et al.* (2003) and Lopez-Oceja *et al*. (2016), have used universal primers for species identification. All these involve the use of specific primers for amplifying the target gene and further employing sequencing for species confirmation.

In this case, attempts were made to explore the possibility of using random decamers to generate species/genus-specific amplicons. Such amplicons could ideally represent unique segments of DNA which can then be used for generating species/ genus-specific de novo primers. With this objective, the DNA from tiger, lions, leopard, jaguar, sloth bear, black bear, wolf, jackal and dhole was analyzed using RAPD-PCR. The decamers (AP7 and AP17) revealed DNA fingerprint patterns in all the species under study.

AP7 primer produced polymorphic fragments ranging from 200 bp to 1000 bp between the species. Likewise, the AP17 primer produced polymorphic fragments ranging from 250 bp to 850 bp. For the felid species, both the decamers (AP7 and AP17) produced monomorphic bands with distinct bright amplicons of 500 bp and 450 bp respectively. The amplicons generated were unique to the feline species. Such monomorphic amplicons generated can represent a conserved region among the felid species and primers designed based on the sequences could find utility in the targeted generic detection of *Panthera* DNA. However, this needs further confirmation. The AP7 primer generated two faint but distinct amplicons in lion samples which were unique and this could also be specific to lion species.

For the bear species, the polymorphic fragments generated by the AP7 primer were very distinctive. Two bright amplicons corresponding to 400 bp and 800 bp were observed in both the bear species. These amplicon sizes could also be species-specific, as these were not observed in other samples. Thus, they can be a potential target for designing specific *Ursa* primers. However, the AP17 primer revealed polymorphic fragments with unique amplicons corresponding to 400 bp and 450 bp in sloth bear and black bear respectively. The fingerprint pattern observed in bears was distinct from the felid and canid species.

In the canid species, the AP7 primer revealed distinct fingerprints. In the wolf and jackal, monomorphic bands were generated with product sizes of 600 bp and 500 bp. However, in dhole, polymorphic fragments were generated with an amplicon of greater intensity at 1000 bp size. This pattern was unique to dhole and was not evident in other species. The AP17 primer produced polymorphic fragments in all the canid species that were distinct from the patterns generated for other DNA. In addition, the pattern of the polymorphic fragment generated by the AP17 primer was distinctive (500 bp, 600 bp and 620 bp) among the canid species. The fragments generated were unique and it is possible that these can be species-specific products. The fingerprint pattern observed in canids is distinctive and unique with both primers.

The monomorphic fragments generated in this study can be representing a conserved region and these fragments can be further sequenced to confirm whether these fragments are genus-specific or species specific. Sequences obtained from individual fragments can then be used to generate primers for PCR-based diagnosis. In the study conducted by Sreekumar *et al.* (2003), RAPD generated polymorphic fragments were used to design specific primers for differentiating the coccidian species *Neospora caninum* and *Hammondia heydorni*. Similar studies can be explored with the available results to design de-novo primers for species identification.

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