DEVELOPMENT OF TIGER-SPECIFIC PCR PRIMERS AND THEIR APPLICATION IN CONSERVATION AND FORENSICS

4.1 Introduction

Reliable identification of scats of sympatric carnivores is very important in wildlife management and conservation. This becomes difficult if there are more than one secretive sympatric carnivore coexisting in the same area. The tiger is sympatric in distribution to the leopard Panthera pardus and the wild dog/dhole Cuon alpinus all over its habitat in south Asia. Scat samples are used in studies of the dietary preference of the three sympatric carnivores to study prey preferences and partitioning of ecological niches (Karanth and Sunquist 2000). Behavioral and morphometric signs like coiling of scat are used in order to distinguish between the scats of the two sympatric large felids, the leopard and the tiger (Biswa and Sankar 2002). However, these methods maybe error-prone and subjective. On the other hand, molecular methods of distinguishing faecal samples of sympatric carnivores are more successful than morphological methods (Davison et al. 2002). The method commonly employed is either PCR amplification of mitochondrial DNA and sequencing the PCR products (Farrell et al. 2000; Verma and Singh 2003) or PCR amplification of mitochondrial DNA followed by restriction digestion based on species-specific restriction sites (Livia et al. 2007; Vercilli et al. 2004). Species-specific methods have also been used for distinguishing the scat samples of sympatric carnivores (Palomares et al. 2002; Dalèn et al. 2006). A genetic method of identifying the scat and hair samples of tigers from those of their sympatric carnivores was developed in the current study. This method is based on the ARMS-PCR or Amplification Refractory Mutation System-Polymerase Chain Reaction of tiger-specific variations in the mitochondrial cytochrome b gene. An ARMS-PCR targeting such regions provides reliable and convenient method to distinguish tiger samples from others. These primers can also be used in the forensic identification of confiscated biological samples of suspected tiger identity.
4.2 Materials and Methods

4.2.1 Development and validation of tiger-specific mitochondrial cytochrome b primers

Mitochondrial cytochrome b sequences of the Bengal tiger, *Panthera tigris tigris* (AF053019-25), its sympatric carnivore leopard, *Panthera pardus* (AY 005809) and some of the animals preyed upon by tiger namely sambhar *Cervus unicolor* (AF423201), barking deer *Muntiacus muntjac* (AY225986), wild pig *Sus scrofa* (AY237529), hog deer *Axis porcinus* (AY035874), Indian bison *Bos gaurus* (AF348593), spotted deer *Cervus axis* (AY182236), domestic goat *Capra hircus* (AB110595), domestic buffalo *Bubalis bubalus* (D82894) were downloaded from GenBank. All the sequences were aligned in ClustalX 1.8 with *Panthera tigris tigris* (AF 053018) as the reference sequence, to identify nucleotides that were found exclusively in tiger but not in the other species. ARMS (Amplification Refractory Mutation System) PCR primers were designed such that the 3’ ends of the forward and reverse primers targeted bases specifically found in tiger and not in sympatric carnivores or prey animals. The primer sequences are:

TIF: 5’- ATAAAAATCAGGAATGGTG -3’
TIR: 5’- TGGCGGGGATGTAGTTATCA-3’

Initially these primers were tested by *in silico* PCR on GenBank sequences with the Amplify 1.2 software (Engels B, Department of Genetics, University of Wisconsin, Madison WI 5706) following which they were PCR-amplified with DNA of some animals namely, Bengal tiger (*Panthera tigris tigris*), Siberian tiger (*P. altaica*), leopard (*P. pardus*), lion (*P. leo*), clouded leopard (*Neofelis nebulosa*), domestic cat (*Felis catus*), wild dog (*Cuon alpinus*), wolf (*Canis lupus*), jackal (*Canis aureus*), goat (*Capra hircus*), wild pig (*Sus scrofa*), Indian bison (*Bos gaurus*), Spotted Deer (*Cervus axis*), Sambar (*C. unicolor*), human (*Homo sapiens*).

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obtained from the ‘DNA Bank’ at the Centre for Cellular and Molecular Biology (CCMB), India.

4.2.2 Field Work for sample collection

For faecal sample identification trails of wild tigers, eight carnivore faecal samples were collected from the Nagarjunasagar-Srisailam Tiger Reserve (NSTR), Andhra Pradesh over a period of four days during the Tiger Census conducted by the Andhra Pradesh Forest Department in January 2002. One of the samples was known to have come from a tiger that was seen defecating near a cattle kill site by tribal villagers.

In a preliminary study at Kawai Wildlife Sanctuary, scat samples (n=15) which were presumed to be tiger scats based on morphology were collected during the Tiger Census of 2005. Beat locations where the samples were collected were Dongapalli, Gangapur, Islampur, Allampalli, Errabandalu, Kalpakuntagutta, Pattaniladaram, Budenragadi, Karrebandalu, GGpur, Kaponiboru.

4.2.3 Forensic analysis with tiger-specific primers

In a case study, four pieces of animal flesh were forwarded to us by the Forest Department of a Tiger Reserve in Central India. These were obtained during a routine patrolling and were suspected to that of a tiger, apparently killed and skinned. Formalin-preserved samples of the same were forwarded to us for forensic identification.

In two separate cases, a total of five skin samples suspected to be that of tiger were forwarded for molecular forensic identification (Figure 4). In both cases, DNA was extracted and PCR amplification was carried out both with TIF/TIR primers and universal mcb cytochrome b primers in separate reactions.

4.2.4 PCR amplification and data analysis

PCR amplifications with tiger-specific cytochrome b primers TIF/TIR was carried out in 25 µl reactions with the following final concentration: 1x PCR Buffer II (Applied Biosystems), 1.5 mM MgCl₂ (Applied Biosystems), 0.15 mM dNTPs, 1x BSA (New England Biolabs), 1U
AmpliTaq Gold, 5 μl each of primers TIF and TIR and 3 μl template DNA. PCR reactions were carried out in MJ Research PTC-200 Thermal Cycler with the following conditions: initial denaturation of 95°C for 10 minutes, 40 cycles of 95°C for 45 seconds, 59°C for 30 seconds (Ramp: 0.5 °C/Sec), 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes.

Positive amplicons that were obtained in the tiger-specific PCR assay with TIF/TIR with DNA isolated from the eight carnivore scat samples collected from Nagarjunasagar Srisailam Tiger Reserve were sequenced on an ABI 3730 Automated DNA Sequencer. Sequences obtained were aligned using ClustalX 1.2 with GenBank reference sequence of tiger (AF053019). Some animals that are the prey species of tiger and leopard, the sympatric felid, were also used in the alignment. The sequences were formatted using GENEDOC software. A phylogeny analysis of aligned sequences was done with MEGA v3.1 software (Kumar et al. 2004). NJ tree was constructed with the sequences using Interior Branch Test of Phylogeny with default values for the number of replicates.

Forensic samples were PCR amplified with the tiger-specific primers as well as universal mcb primers to confirm whether they were indeed that of tiger identity.

4.3 Results
4.3.1 Development and validation of tiger-specific PCR assay
Multiple sequence alignment with Clustal X of cytochrome b from various species showed tiger-specific variations at position 636 and 759 of the tiger mitochondrial DNA and not in the other animals (Figure 5). Initially an in silico PCR was performed using the software Amplify 1.3 on GenBank sequences of tiger and sympatric carnivores as well as their prey. A PCR product of size 162 bp was predicted in the tiger sequences but not in those of other animals tested.
Skin samples of suspected tiger identity were analyzed in a tiger-specific PCR assay in order to confirm whether the samples were tiger in origin.
Figure 5
Sequence alignment of some of the prey species of tiger, the extant subspecies of tiger and other carnivores.

Arrows indicate the tiger-specific variation at positions 636 and 759 that were used to design the primers TiF/R for tiger-specific diagnostic assay for tiger faecal sample identification.
PCR amplification was carried out with the DNA of captive animals namely Bengal tiger (*Panthera tigris tigris*), Siberian tiger (*Panthera tigris altaica*), leopard (*Panthera pardus*), Wild dog (*Cuon alpinus*), clouded leopard (*Neofelis nebulosa*), Wolf (*Canis lupus*), Domestic cat (*Felis catus*), Sambar (*Cervus unicolor*), spotted deer/chital (*Cervus axis*), wild pig (*Sus scrofa*), Jackal (*Canis aureus*), Indian Bison (*Bos gaurus*), Buffalo (*Bubalis bubalus*). Amplicons were obtained only in tiger DNA of both Bengal and Siberian tigers, as predicted in the *in silico* PCR (Figure 6a and Figure 6b).

### 4.3.2 Scat sample analysis

DNA was isolated from eight carnivore samples collected from Nagarjunasagar Srisailam Tiger Reserve and PCR amplified with the primers TIF/TIR. Two samples were positive for tiger, one of which was known to have come from a tiger near a cattle kill site. The amplicons were sequenced and aligned with sequences of some of the animals are the prey of tiger and sympatric carnivores. NJ tree was constructed (Figure 7); the sequences cluster with GenBank tiger sequences, thus proving conclusively that the samples were of tiger.

Fifteen scat samples were collected in two successive occasions in Kawal Wildlife Sanctuary during the Tiger Census in 2005. Beat locations where the samples were collected were Dongapalli, Gangapur, Islampur, Allampalli, Errabandalu, Kalpakuntagutta, Pattaniladaram, Budenragadi, Karrebandalu, GGpur, Kaponiboru. Samples from Kalpakuntagutta, Islampur and Gangapur were positive in the tiger-specific PCR assay (Figure 8). This was indicative of the persistence of tiger even in certain areas of this forest in spite of severe human disturbance (Figure 9).

### 4.3.3 Forensic Identification of samples of suspected tiger identity

PCR amplification with the primers TIF/TIR of DNA isolated from the formalin-preserved flesh samples forwarded by the forest department in Central India turned out to be that of tiger (Figure 10) as suspected initially. Whereas none of the five skin samples that were suspected to be of tiger identity were tiger in origin (Figure 11). No amplicons were obtained on using the tiger-specific primers. A BLAST analysis of the PCR products of the *mcB* PCR products revealed that three of the samples were of domestic dog and the rest were of domestic cow.
Figure 6a

A representative gel picture showing the specific amplification of only tiger DNA with the tiger-specific PCR mitochondrial cytochrome b primers

Lane 1: 100bp ladder, Lane 2: Negative control, Lane 3: Panthera pardus, Lane 4: Cuon alpinus; Lane 5: Neofelis nebulosa; Lane 6: Cervus unicolor; Lane 7: Panthera tigris tigris; Lane 8: P. t. altaica; Lane 9: Bos gaurus; Lane 10: Cervus axis; Lane 11: Sus scrofa; Lane 12: Cervus axis.

PCR amplification with universal ‘mcb’ primers of all animals tested rules out the possibility of false negatives in PCR with tiger-specific primer pair (TIF/TIR). Amplification with primers TIF/TIR and mcb primers was carried out in separate reaction though the PCR products were loaded onto a gel together.
Figure 6b

Gel profile showing the specific amplification of random samples of captive tiger DNA

Panel 1:
Lane 1: 100 bp ladder, Lane 2: negative control, Lane 3 to 12: Captive tiger DNA,
Lane 13: Clouded leopard (Neofelis nebulosa),

Panel 2:
Lane 1: 100 bp ladder, Lane 2: Clouded leopard (Neofelis nebulosa), Lane 3 to 7: Leopard (Panthera pardus), Lane 8, 9: Sambar (Cervus unicolor), Lane 10: Chital (Axis axis), Lane 11: Snow Leopard (Panthera uncia)
Figure 7

Neighbour-Joining tree of PCR amplicons positive in the tiger-specific PCR assay

Neighbour-Joining tree of amplicons of two faecal samples from Nagarjunasagar Srisailam Tiger Reserve (NSTR1 and NSTR2). Sequences of amplicons of both samples, which were positive in tiger-specific diagnostic assay, cluster with GenBank tiger sequence proving further that the samples are tiger in origin.
Figure 8

Tiger specific PCR assay to determine the presence of tigers in a disturbed habitat

Lane 1: 100 bp ladder, Lane 2: Negative Control, Lane 10: Positive Control (tiger genomic DNA). Three samples (Lanes 5, 6 and 8) from Kawal Wildlife Sanctuary were positively identified as tiger.
Encroachments and human settlements in the forest are shown as yellow circles and approximate locations of the tiger scat samples are indicated by red circles. The tiger-specific PCR assay showed tiger presence even in disturbed forests and the need to protect and conserve such habitats.
Figure 10
Tiger-specific PCR assay for forensic identification of samples of suspected tiger origin

Lane 1: 100bp ladder, Lane 2: Negative control, Lane 7: Positive control tiger genomic DNA. Three out of the four samples were positive in a tiger-specific PCR, indicating that the samples were tiger in origin as initially suspected.
Figure 11

Tiger-Specific PCR assay of skin samples of suspected tiger identity

Lanes 1 to 5: PCR amplicons of skin samples suspected to be that of tiger with universal mcb primers. None of the skin samples that were suspected to be of tiger amplified with tiger-specific primers although they amplified with the universal mcb primers.

Sequencing and subsequent BLAST analysis of the universal mcb PCR amplicons showed that the samples were from domestic dog and cow, painted to look like tiger skins.
4.4 Discussion

Traditionally, scat samples of carnivores have been used for analyzing the dietary preferences. In studies of dietary preferences by tiger and its sympatric carnivore the leopard, morphological methods have often been used like the diameter of sample, degree of coiling etc. However, these methods may often be subjective and sometimes even trained naturalists have failed to identify the samples accurately (Davison et al. 2002). The tiger-specific PCR assay developed in this study can help to identify tiger scats before proceeding for further downstream analysis like microsatellite DNA analysis and identification of the sex of the samples.

In most tiger habitats that are outside of protected Tiger Reserves, tigers may exist in low densities. Such low-density areas now cover more than 90% of the tiger distribution range (Karanth et al. 2002). For studies of tiger presence in such areas, signs surveys are generally used like presence of pugmarks and scrapes. In the light of human disturbance like cattle grazing in some protected areas such tiger signs may get obliterated. Therefore, it may be more feasible to use more than one method of obtaining evidence of tiger presence. A method that makes use of the tiger-specific PCR for amplification of faecal DNA in addition to the survey methods can provide definite and direct evidence as to the presence of tiger in low density areas.

The Kawai Wildlife Sanctuary is a protected area in Andhra Pradesh but is not a designated Tiger Reserve where tiger presence has been recorded in the past. Kawai Wildlife Sanctuary has been identified as a Type III Tiger Conservation Unit (TCU), with protected area of 1057 km$^2$ within a TCU area of 4307 km$^2$ (Wickramanayake et al. 1999). This Sanctuary comprises of several human settlements and is subject to severe human disturbance due to activities like bamboo felling, cropping of forest produce, cattle grazing. However large tracts of this forest are still pristine with minimal human disturbance. The tiger habitat type in this protected area is mainly of the tropical moist deciduous type with teak *Tectonia* sp the predominant plant species mixed with bamboo, *Terminalia, Cassia* etc.
Presence of tigers has been recorded here previously though direct sightings are becoming increasingly rare. Previous attempts at camera trapping to determine the presence of tigers have not been successful due to human disturbance (Siddiqui I, personal communication). Moreover camera trapping is advised against in those areas where tigers are low in density as in Kawal; instead it has been recommended that tiger abundance be estimated in such areas from presence of tiger signs like scats or tracks (Karanth and Stith 1999). Tiger scats are used in sign surveys to detect for presence of/or abundance estimates of tigers in protected areas (Karanth et al. 2002). In the jungles of India and most of South Asia, the tiger is sympatric to other carnivores like leopard Panthera pardus and Wild dog Cuon alpinus. Tiger scats are distinguished from those of the sympatric carnivores by parameters like morphology of bolus (Biswas and Sankar 2002) or by signs associated with defecation behaviour like scrapes (Schaller 1967). Using the PCR assay for scat identification if used in conjunction with the standard presence/absence surveys can give reliable and reasonable estimates of area occupied by tigers in disturbed habitats like Kawal Wildlife Sanctuary (Naidu 2007) and help in conservation planning and management.

This PCR assay developed here can also be used for forensic identification of samples of suspected tiger identity. DNA extracted from forensic samples and non-invasively collected samples like faecal samples and hair contains poor quality if not degraded DNA and therefore primers targeting smaller PCR amplicon size would amplify better.

A disturbing fact that arises from the case samples of Central Indian forest is the poaching of tigers from National Parks in India. Poaching statistics compiled by the Wildlife Protection Society of India put the number of tigers killed from 1994 to 2006 at 797 (http://www.wpsi-india.org/statistics/index.php). Added to that is a new problem in the form of trade in fake tiger skins in which domestic dog, goat or cow skins are painted to resemble tiger skins for sale. According to the Wildlife Protection Society of India, the number of fake tiger skins in the market has increased substantially in the last couple of years (http://www.5tigers.org/news/CatNews/No.26/cn26p08.html). This leads to a considerable wastage of time and expense on the part of law enforcement officials in accurately identifying such samples before booking charges on the accused. The tiger-specific PCR assay described
here can be used to identify with just a single PCR reaction whether the suspected samples are that of tiger in origin or fakes based on the presence or absence of an amplicon in the PCR, thus saving considerable time. Moreover as the tiger-specific PCR does not require sequencing, it is cheaper and can be carried out even in laboratories that do not have access to DNA sequencers. DNA of samples of suspected tiger identity should be amplified in parallel with ‘universal’ primers in order to rule out the possibility of false negatives in PCR. Though some studies have used microsatellite markers to distinguish samples of large cat species (Singh et al. 2004) however methods based on mitochondrial, which are in higher copy number in a cell compared to nuclear DNA provides a greater advantage. The probability of amplifying a mitochondrial fragment is therefore higher than a nuclear gene, especially when working with sub-optimal sources of DNA like scat samples or forensic samples in order to determine whether a given sample is tiger in origin.

The usefulness of the tiger-specific PCR assay could be compromised by the presence of intra-specific polymorphisms, especially in leopard, in studies of sympatric leopard and tiger. However as the primers TIF and TIR are based on two variations in the tiger mitochondrial cytochrome b gene, the possibility of having variations at both these sites in leopards at the same time would be low. Thus a variation at either of these sites would result in non-amplification but not in misidentification of leopard scats as tiger.