GENETIC ANALYSIS OF PANTHERA PARDUS AND PANTHERA TIGRIS BY mtDNA PCR

T H E S I S

Submitted

in partial fulfillment of the requirements for the Degree of

M A S T E R OF V E T E R I N A R Y S C I E N C E

IN

ANIMAL BIOTECHNOLOGY

BY

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DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled "**GENETIC ANALYSIS OF PANTHERA PARDUS AND PANTHERA TIGRIS BY mtDNA PCR"** or part thereof has not been submitted for any other degree or diploma of any university, nor the data have been derived from any thesis/publication of any university or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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Miss **ACHARYA POOJA SANJAY** has satisfactorily prosecuted her course of research for a period of not less than one semester and that the thesis entitled, "**GENETIC ANALYSIS OF PANTHERA PARDUS AND PANTHERA TIGRIS BY mtDNA PCR"** submitted by her is the result of research work is sufficient to warrant its presentation to the examination in the subject of **ANIMAL BIOTECHNOLOGY** for the award of **Master of Veterinary Science** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

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CERTIFICATE

This is to certify that the thesis entitled, "**GENETIC ANALYSIS OF PANTHERA PARDUS AND PANTHERA TIGRIS BY mtDNA PCR**" submitted by

Miss **ACHARYA POOJA SANJAY** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **Master of Veterinary Science** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

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If you dilute your duty, you will have to pollute your-self……

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Place: Nagpur ***(Acharya Pooja Sanjay)***

Date:

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µl	Microlitre	Bp	Basepair
P. pardus	*Panthera pardus*	Mt	Mitochondria
P. tigris	*Panthera tigris*	Mg	Milligram
ATPase 8	ATP synthesis protein 8	Ml	Milliliter
Cyt. b	Cytochrome b	Min	Minutes
GEL-Doc	Gel Documentation	mM	Milimolar
AGE	Agarose gel electrophoresis	Sec	Seconds
Ppo-PF	*Panthera pardus* – primer forward	Etc	Ethically all
Ppo-PR	*Panthera pardus* - primer reverse	&	And
Pta-PF	*Panthera tigris* – primer forward	UV	Ultraviolet
Pta-PR	*Panthera tigris* – primer reverse	µl	Microlitre
mtDNA	Mitochondrial DNA	OD	Optical density
PCR	Polymerase Chain Reaction	%	Percentage
DEPC	Diethyl-pyrocarbontes	^{o}C	Degree Celsius
TBE	Tris Boric acid EDTA	Viz.	It is permitted to see
EDTA	Ethyl diamine tetra acetic acid	V	Volts
WRTC	Wildlife Research Training Center	Tm	Melting temperature
dNTPs	Dinucleotide triphosphates	M	Molar
rRNA	Ribosomal Ribonucleic Acid	RFLP	Restriction Fragment Length Polymorphism
SNP	Single nucleotide polymorphism	w/v	Weight/Volume
CITES	Convention of International Trade on Endangered Species	v/v	Volume/Volume
NFW	Nucleus Free water	%	Percent
TE	Tris EDTA	Conc.	Concentration
Rpm	Revolution per minute	WRTC	Wildlife Research and Training Centre
FDCM	Forest Development Corporation of Maharashtra		

INTRODUCTION

India is rich in bio-diversity, having special position in field of wildlife conservation in the world. It is rich in having natural beauty in terms of big cats with very unique roaring sound. It has long history of heritage, tradition of animal conservation and boasts the variety of species and organisms. Wildlife is one of the cultural beauty in India, it offers an opportunity to art enthusiastic persons and its exception with wildlife conservation. The wildlife interested persons can study their behavior, habitat and interactions of wildlife by walking and observing in and around area of them. Wildlife comprises of variable types of wild animals of different families viz., Carnivores which involves Felidae, Canidae and Ursidae etc. Wildlife forensics is the story which originates from crime scene and concludes in the court (http://www.pib.nic.in/newsite/mbErel.aspx?relid=105134).

The tiger and leopard belongs to kingdom Animalia, Family Felidae, genus Panthera, species *Panthera tigris* and *Panthera pardus* respectively. Whenever, we talk about wild-animal, it's the topic of prestige and pride but their population monitoring is difficult as they live in dense forest areas. Human – wildlife conflicts is defines as, Interaction between human and wildlife where negative consequences whether perceived or real, exists for one or both the parties when action of one party has adverse effect on other party (Conover, 2001; Decker *et al.,* 2002). So molecular forensics helps to solve the cases of human wildlife conflicts.

In cases of Human Wildlife Conflict and wildlife forensics, the powerful method for detecting the origin and identification of endangered species is genetic analysis of that respected sample. For species identification the prerequisites are availability of the sample, small genetic difference in species should be there, to make sense. In general wild species identification is done by genes in Mitochondrial DNA (mtDNA). There are no. of copies of mitochondrial genes in cell than genes in nuclear DNA (Burgener *et al.,* 1998).

There are techniques for wildlife species identification viz., Morphological, footprints, serologic techniques, molecular techniques. Molecular techniques are more reliable as compared to other techniques as they give more specific results. DNA fingerprinting (Wan *et al.,* 2003), Polymerase Chain Reaction – Restriction

Fragment Length Polymorphism (PCR-RFLP) (Nagata *et al.,* 2005), Real-time PCR and sequencing (Gupta *et al.,* 2011). Real time PCR (Wetton *et al.,* 2004; Waits *et al.*, 2005). It has many applications including forensics, evolution studies, monitoring of illegal animal and their product trades (Fajardo *et al.,* 2010).

The genetic techniques are all based on PCR, viz. using species specific primers (Sugimoto *et al.,* 2006), DNA sequencing of PCR products (Farrell *et al.* 2000) or PCR-RFLP (PCR-Restriction Fragment Length Polymorphism), which is a combination of PCR followed by digestion of the PCR product with diagnostic restriction enzymes to yield species-specific banding patterns (Cossíos *et al.,* 2006). DNA sequencing gives very specific results but its time consuming and costly as compared to gel based screening and PCR-RFLP. Majority felid species are elusive and rare, so population monitoring is done by genotyping of scat DNA (Mondol *et al.,* 2009) and dietary study (Farrell *et al.,* 2000).

The most commonly used molecular method is PCR amplification of mitochondrial DNA and sequencing the PCR products (Farrell *et al.,* 2000) or PCR amplification of mitochondrial DNA followed by restriction digestion based on species-specific restriction sites (Livia *et al.,* 2007). Species-specific methods have also been used for distinguishing the scat samples of sympatric carnivores (Dalen *et al.,* 2006).

Carnivore scats formed the basis of one of easily available noninvasive and humane sample collection technique. It was reported that, scat genetic analysis is one of the robust and accurate method for identification of tiger from degraded scat DNA extracts. The fecal material of leopard and tiger resemble with each other morphologically, hence its hard to differentiate amongst them on basis of fecal samples. Similarly the collection of any other samples like blood, urine, hairs, tissue requires approaching these wild animals and is interfering into their zone and due to strict norms of Wildlife Protection act. The scat sample is one of the most useful easily available sample for investigation. The genetic methods are found useful to differentiate similar scats between closely related species. The mtDNA is found to be more stable than the genomic DNA and it is abundant than gnomic DNA in scat samples. Several researchers have taken the use of mtDNA for identification of the species of animals based on genes located on mtDNA viz., Cox 1, Cytochrome b, ATPase, ND4 loci, 12s and 16s rRNA etc.

In Vidharbha region, several cat families members are being residing in different forest. However, none of the report signifies the genetic analysis of these wild animals. As there are always man and animal conflict of being man eater or so. So the presence of particular animal is to be firmly confirmed to take any further action to avoid the killing of innocent one. Similarly, the wild animal sample collection remains one of trickiest part. however the scat sample can easily obtained by humane methods in captivity as well as in wild. With advances of genetic techniques using the scat samples to identify the species need to be evaluate to enforce the protection of endangered species Hence, the present study was undertaken to develop a rapid and cost-effective protocol for the reliable identification of tigers and leopards scats from sympatric carnivore scats.

Objective:-

1) To carry out genetic analysis of samples by amplification of cytochrome b / ATPase 8 genes from samples of *Panthera pardus* and *Panthera tigris* kept at Wildlife Research and Training Centre, Gorewada, Nagpur.
2) To analyze the sequence of cytochrome b / ATPase gene amplified products and carry out phylogenetic analysis.

REVIEW OF LITERATURE

In India HWC was reported in 32 states and union territories of India viz., Karnataka, Assam, Gujrat are top most reported locations of conflict. The form of conflict includes the human injuries and casualties by tiger and leopard in Karnataka and crop depredation in all three states. HWC in Karnataka is mainly due to elephant crop depredation and human casualties by elephant, tiger and leopard. Assam reported crop depredation by primates, geographical distribution of conflict showed dramatic increase in time during the period 1976e1995. HWC was reported from 11 states and union territories in India, whereas the no. increased in period of 1976 to 2005. Total 88 no. of nine different taxonomic groups were reported to be involved in conflict. Species involved are as carnivores (n ¼ 21), rodents (n ¼ 19), ungulates (n ¼ 17). Presently the main conflict species are Aian elephant, Indian tiger, leopard1996 to 2015 (Shaurabh *et al.,* 2017). Wildcat are secretive in nature, because of that it is difficult to tally and monitor its populations by direct observations. Alternatively, incidental methods were used for studying tigers in the wild, DNA methods based on non-invasive sampling have not been attempted so far for tiger population studies in India.

The principal technologies and application available to wildlife forensic geneticists, focusing on the four most common caseworks includes, species identification, its origin, its purpose, is it captive bred or free range. The conversion of established research tools into forensic identification systems was discussed, explained the need for method validation at each stage of the analytical process, from sample collection to data analysis. The requirement for wildlife DNA forensic analysis to be performed under equivalent quality assurance standards to those of human forensic genetics is highlighted and approaches for the interpretation and presentation of DNA evidence are described. A perspective is provided on the potential for new genetic techniques and their future role in the increasingly complex fight to enforce the protection of endangered species. The review concludes with a number of recommendations for promoting a unified, rigorous approach to the development and application of wildlife DNA forensic techniques (Ogden *et al.,* 2009).

2.1 Sampling in wildlife Species Identification

In wildlife species identification, sampling is one of the fiddly job because norms of Wildlife Protection act, 1972. In wildlife, species identification from non-invasive samples is great challenge to wildlife forensic. Non-invasive method of sampling is method of choice in wildlife species identification hairs, dried scats, bones, claws etc. of known or unknown origin. Nuclear DNA is not stable in comparision with mtDNA so it don't lasts for long duration, hence in most of the cases mtDNA is used in wildlife species identification.

To identify biological specimens from 44 diverse vertebrate animals viz. mammals, birds, reptiles, amphibians and fishes. The partial cytochrome b gene was amplified, derived sequences were used to identify the biological origin of the samples by aligning to cytb gene sequence entries in nucleotide databases. The applicability of this method to the forensic field is demonstrated by simulated casework conditions where different types of samples including problematic specimens such as hair, bone samples, bristles and feathers were investigated to identify the species (Parson *et al.,* 2000).

Wildlife forensic science is a fast growing field with the availability of successful case reports (Verma *et al.,* 2003; Gupta *et al.,* 2005, 2011). Several genetic studies in endangered animal species are based on ability to get more accurate and specific results from very minute amounts of DNA from non-invasive samples viz., scats, hairs, scales, shaded skin, urine etc. Particularly Carnivores are more prone to loss of habitats because of their space and prey requirements, low density occurrence and there forest dependent habitat which provides them sufficient safety (Nowell *et al.,* 1996; Crooks *et al.,* 2002).

mtDNA is Non-recombining, Fast-evolving, Uniparental inheritance, High copy number, Ubiquitous, Simple structure, 37 genes coding for 13 proteins, 22 tRNAs, 2 rRNAs, Gene order conserved, Small size [16.5kb] (Ifumagalli *et al.,* 2015), exonuclease-resistant circular nature, lack of recombination and the maternal mode of inheritance hence comparisons can be made by getting reference sample from maternal side even though the sample are separated by many generations. Nucleic acid-based species identification frequently targets the mitochondrial encoded cytb gene.

2.2 Wildlife Species Identification

The *Panthera tigris* is the largest felid species and a widely recognized symbol of wildlife conservation (Luo *et al., 2004*). Historically tigers are inhabitant of much of Asia, Caspian and aral seas, Southeastern Russia and Sunda Islands (Herrington *et al.,* 1987). Since early 1900s tiger population was reduced due to habitat loss, fragmentation and human persecution from 1000000 in 1900 to fewer than 7000 free ranging individuals (Kitchener *et al.,* 2000). Most population consists below 120 animals, increasing the risk of local extirpation due to demographic and genetic factors (Dinerstein *et al.,* 1997). Mitochondrial gens consist of more sequence diversity, which helps in identification of phylogenetically related species (Girish *et al.,* 2005).

Panthera pardus is the most elusive of pantherine felids (Grassman *et al., 2005*). Analysis of molecular (Wilting *et al.,* 2007) and morphological data (Christiansen *et al.,* 2008) demonstrated that Bornean and Sumatran clouded leopards are clearly distinct from those on the continental mainland (Wilting *et al.,* 2011).

To understand the molecular phylogeny of cytochrome b and 12S rRNA sequences of mitochondrial DNA in Felidae Family. The blood sample of 23 species different was collected and processed for PCR, which found six species were from domestic cat lineage, six from ocelot lineage and other eight from different pantherinae lineage. The spotted hyena (Hyenidae), ring tail mongoose (Herpestidae) and fnaloka fossa fossa (Viverridae) as outgroup. The primers were designed using consensus sequences of human, bovine and murine, PCR was done. Sequencing was done and phylogenetic tree was prepared which found the pairwise same values between felids from 94 to 99%, which helped in understanding intralineage associations but failed to completely resolve interlineage relationships (Masuda *et al.,* 1995).

To identify the predation behavior of small carnivores viz., Puma, Jaguar, Ocelot and crab-eating fox. The fecal samples were collected during a radiotelemetry study of pumas and jaguars at Hato Pinero, Cojedes, Venezuela, in 1996. Analysis of fecal samples of Puma (*Puma concolor*), jaguar *(Panthera onca)*, ocelot *(Leopardus pardalus*) and crab-eating fox (*Cerdocyon Thous)* were done to identify their prey animals. The carnivore specific primers were designed

for cytochrome b gene and PCR was done which successfully amplified 146 bp amplicon. Sequencing of amplified products were done, which reveals the detection of Domestic dog (*canis familaris)*, Wild boar (*sus scrofa*) and ocelot (Farrell *et al.,* 2000).

To identify the offcuts of endangered animals and endemic animals in Taiwan and for conservation of animals. Study was done with 19 species viz., formosan gem-faced civet, leopard cats, tigers, claudeed leopards, lion, Formosan muntjac, Formosan sika deers, Formosan sambars. Formosan serows, water buffalo, Formosan pangolins and Formosan macaque. Control animals were domestic dog, domestic cat, domestic sheep, domestic cattle, domestic pig and humans. The available forms of samples were collected and processed for PCR using universal pair of cytochrome b. The amplified PCR products were sequenced and obtained sequences were further cross matched with available reference sequences which revealed the successful species identification (Hsieh *et al.,* 2001).

To identify the endangered species Siberian tiger (Panthera tigris altaica) and Amur leopard (Panthera pardus orientalis) at eastern Russia due to habitat loss and poaching. The species specific primers were designed by alignment of cytochrome b sequences of leopard, tiger and some other carnivore species viz., cat, dog, red fox, sika deer, roe deer, wild boar, red backed hole and human. Interspecific sequence difference were selected for tiger–leopard-specific-primer and primers were designed PantheraL1/ PantheraL2/ PantheraH1 to amplify only tiger and leopard. PCR was done with designed and published primers which revelaed, amplification of designed (280) and published primer (423 bp, 424 bp). Furthermore the amplified products were digested with Hinf I and validation of samples were done, which helped in differenciation of tiger and leopard (Nagata *et al.,* 2005).

Amur leopard *Panthera pardus orientalis*is most endangered leopard in the world, they are mainly distributed in Primoryekrai. Sympatrically amur leopard found to be with *Panthera tigris tigris* in Primoryekrai. To identify these endangered species the species specific primers were designed using amur leopard and Siberian tiger cytochrome b sequences. The obtained sequences were aligned with leopard cat sequences and primers were designed as PpoF/PpoR, PtaF/PtaR. The PCR revealed amplification with respected size. To

check specificity of primers, sequencing was done, which showed the primers are species specific they do not amplified the DNA of prey species (Sugimoto *et al.,* 2006).

For detecting feasibility of non-invasive scat samples for evaluation and monitoring of tigers by genotyping. The random non-invasive fecal sampling was done at mudumalai and Biligiri Rangan Temple, Wildlife Sanctuaries. The tiger specific primers were designed by identifying unique nucleotide sequences in tiger viz., guanine at 636 and thymine at 759 positions respectively. The designed primers were amplified 162 bp fragment of cytochrome b gene in silico PCR. The two samples collected from Nagarjunasagar-Srisailam Tiger Reserve (NSTR) were positive for tiger, subsequently sequencing was done. The sequences were clustered with *Panthera tigris* and phylogenetic tree was prepared, which confirmed the samples were of tiger origin (Bhagavtula *et al.,* 2006).

Conservation and management of elusive and rare animals needs accurate data on presence or absence, in such cases molecular based techniques proves invaluable with noninvasive sampling method. Whereas noninvasive sampling gives low DNA concentration, which might results in false negative species identification. For increasing specificity of results the tiger specific primers were designed. PCR was done and cross amplification of designed primers were checked with other carnivores and herbivores from identified captive individuals. This concludes that, the designed primers have species specificity to tigers (Mukherjee *et al.,* 2007).

The endangered *Panthera uncia (*snow leopard) which occurs in rugged, high altitude regions of Central Asia. Non-invasive scat sampling was done at three different geographical regions viz., one at Ladakh, two at Dulan County, China and three at South Gobi. The carnivore specific primers were used for PCR to amplify 148 bp sequence of cytochrome b. Subsequently sequencing was done, which revealed large-scale noninvasive studies that will provide information critical for conservation of snow leopards (Jeneka *et al.,* 2008).

Molecular markers are crucial tools for identification of species and determining the genetic variation, biodiversity with high levels of accuracy and reproducibility. Widely used molecular markers with decreasing order of

conserved sequences are 12S rDNA> 16S rDNA> cytochrome b > control region (CR) amongst them 12S rDNA is highly conserved and highly variable region is control region. Whereas the commonly used nuclear markers for DNA fingerprinting are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites. These helps in application of molecular markers for biodiversity analysis of wildlife animals (Arif *et al.,* 2009).

Muscle samples of *P. pardus*, *P. tigris*, and skin of *P. uncia* were collected from Ningguo, Anhui province in China. 34 pairs of primers using Oligo 6.0 were designed and the samples were processed for PCR reactions, Sequencing were done. The structures of the genomes were highly similar to *Felis catus*, *Acinonyx jubatus*, and *Neofelis nebulosa.* The phylogenies of the genus *Panthera* were concluded from two combined mitochondrial sequence data sets and the complete mitochondrial genome sequences, by ML (maximum likelihood) analysis. The results showed that *Panthera* was composed of *Panthera leo*, *Panthera uncia*, *Panthera pardus*, *Panthera onca*, *Panthera tigris*, and *N. nebulosa* which was included as the most basal member (Lei *et al.,* 2011).

Noninvasive genetic sampling methods to study wild populations of multiple, co-occurring, threatened felids. This is especially important for molecular scatology studies occurring in challenging tropical environments where DNA degrades quickly and the quality of fecal samples varies greatly. Additionally reliability for species and individual identification using DNA from faeces of wild felids detected by a scat detector dog across Belize in Central America (Claudia *et al.,* 2014).

The complete mitochondrial genome of sequence 16,859 bp of Indian clouded leopard (*Neofelis nebulosa*) has been sequenced using next generation sequencing technology Torrent PGM platform. The complete mitochondrial genome sequence of clouded leopard consists of 13 protein-coding, 22 tRNA, and two rRNA genes and a control region (CR). The base composition of the mitochondrial genome of clouded leopard is as follows: A, 5362 bp (31.8%); C, 4560bp (27.0%); G, 2475 bp (14.6%); T, 4462 bp (26.4%). Most of the genes have ATG initiation codons, except ND1, ND2, ND3, ND4, ND6, and CYTB (ATA start codon). The phylogenetic position of clauded leopard was identified (Tabasum *et al.,* 2016).

The charismatic animals viz., Bengal tiger and Snow leopard were characterized as flagship and umbrella species hence they are center of many conservation programs. The putative tiger, leopard samples were collected from CNP and Annapurna conservation area of Nepal. For species identification, PCR of mitochondrial cytochrome b gene targeting 162 bp and 150 bp for tiger, snow leopard was done using species specific primers. The tiger and snow leopard negative samples were further analyzed for common leopard by using common leopard specific primers targeting NADH4 region of mtDNA. Furthermore, the samples which were negative for Bengal tiger, snow leopard and common leopard were processed for carnivore identification using carnivore specific primers targeting 146 bp sequence of cytochrome b. The carnivore positive PCR products were sequenced, which showed, maximum identity 95% and query coverage above 95% (Karmacharya *et al.,* 2016)

The northern flying squirrel (*G. sabrinus*) and the southern flying squirrel (*G. volans*) comprise together to form *Glaucomys* (New World flying squirrels). To identify their divergence time control region and cytochrome b gene was amplified and phylogenetic tree was prepared, which showed *G. volans* and Continental *G. sabrinus* are actually sister taxa that diverged from one another more recently than either did from Pacific Coastal *G. sabrinus*. The given observations provide valid evidence for third previously unrecognized species of North American flying squirrel, whose geographic range extends from southern British Columbia along Pacific Coast to southern California. *Glaucomys oregonensis* (Bachman, 1839), whose type locality is in Oregon, is the senior available name for this taxon. This newly identified species had given the common name “Humboldt’s flying squirrel.” (Arbogast *et al.,* 2017).

Avian species identification and differentiation is important step in conservation, taxonomic, forensic, legal and other ornithological interventions. To identify some avian species i.e. Chicken (*Gallus gallus*), Muskovy duck (*Cairina moschata*), Japanese quail (*Coturnix japonica*), Laughing dove (*Streptopelia senegalensis*), and Rock pigeon (*Columba livia*) by application of molecular approach. Genomic DNA was extracted from blood samples and partial sequence of the mitochondrial *cytochrome b* gene (358 bp) was amplified and sequenced using universal primers. The obtained sequences were compaired with reference sequences which revealed similarity percentage about 88.60% to

100% betwee*n* the avian *species*, which concluded that, the amplification of partial sequence of mitochondrial *cytochrome b* gene proved to be specific for identification of an avian species (Awad *et al.,* 2018)

To identify and characterize the phylogenetic relationship amongst the wild boars from Argentina with those from Uruguay, Europe, Asia, and the Near East, along with diverse domestic pig breeds in order to corroborate the historical information about the origin of the local boar populations. The cytochrome b and control region were used to amplify species which revealed, that the most of the Argentinian wild boar populations descend from European lineages. The study concluded that genetic diversity in Argentinian populations was lower than Europe and Uruguay interpreting that wild boar in Argentina has experienced minor genetic introgression from domestic pigs, representing in this sense a reservoir of the original wild boar genetic variability (Sangua *et al.,* 2018).

2.3 Species Identification in Forensic cases

Species identification from meat and skin samples were done by using southern blotting and hybridization techniques during the illegal trade and poaching of Chinese water deer (Fang *et al.,* 2003).

The polymerase chain reaction (PCR)-based approach, which without knowing the history of a forensic sample, is able to reveal whether the source of the sample is human or animal. 221 animal species, cytochrome b sequences were aligned together to design novel universal primers (mcb 398/mcb869). The mammals, rodents, carnivores (domestic dog), cetacean, ruminants, pig, bird reptiles etc. about 221 species were identified. Sample collected from one crime scene reveals the identification of Cervus axis. The designed primers are novel to cytochrome b gene of all species (S.K. Verma *et al.,* 2003).

In wildlife crimes STRs are used for individual species identification of different animals in case of illegal trade and poaching. Wild Boar was identified from Blood stains present on knife and carcass (Lorenzini *et al.,* 2005).

To identify the illegal trades 5 animal specimens were received from Council of Agriculture (COA) for species identification. The musty specimens two furs, one penis, one testis, section of organ. The DNA samples were processed for PCR of cytochrome b using designed universal cytochrome b primers

L14724/H15149 and amplified samples were sent for sequencing. Then sequences were compared with registered in DNA bank, which showed 99.7% similarities to registered sequences. This confirmed that the samples were not belongs to illegall trades (Tsai *et al.,* 2007).

In India during 2000 in zoological parks, a young tigress was killed for its claws. A enclosed claw and decomposed tiger hide were grasped from the blamed in 2005. Biological samples of the victim tigress was not available for further forensics examination, hence DNA samples of the biological parents and a male sibling were used to form the identity of the claw using STRs and mitochondrial DNA markers (Gupta *et al.,* 2011).

The geographical origins of clutched ivory of African elephants were identified from Tusks and Hankos respectively (Wasser *et al.,* 2007; Wasser *et al.,* 2008). To detect geographical origin of species in illegal animal smuggling cases, like Chimpanzees and Moose STRs were used (Ghobrial *et al.,* 2010; Ball *et al.,* 2011 resp.)

To protect the endangered animal species, to main biodiversity, poaching, illegal trades. In different wildlife trades, species identification plays important role, so its need to develop authentic and reliable methods. To detect the spcificity of species specific primers, the research was made. The samples of tiger, leopard, wolf and jackel were processed for species specific PCR, which successfully amplified target species. The study reveals species specific primers would be of choice in species identification (Salankar *et al.,* 2012).

2.4 Species Identification of Confiscated scat samples

Officials of Indian zoo claimed that, one animal possibly carnivore is periodically visiting the zoo which creates the panic to zoo and near villages. To identify confiscated species, collection of fecal material of carnivore animals viz., tiger, clouded leopard, lion, white tiger, snow leopard as a reference samples and confiscated fecal material of unknown animal were done. PCR was done, amplified products were sequenced and further analyzed. The sequence of confiscated species matches with known leopard sequence which reveals, the visiting animal was leopard (Verma *et al.,* 2003).

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Place and facilities:

The present research work was performed with the facilities provided at Wildlife Research and Training Centre (WRTC), Gorewada, Nagpur in collaboration with Department of Veterinary Microbiology and Animal Biotechnology, Nagpur Veterinary College, Nagpur.

3.1.2 Details of the samples used in the study:

With prior permission as per the MOU of FDCM Ltd., Nagpur & Maharashtra Animal & Fishery Science University, the coded, 24 fecal samples (scat) were received from Wildlife Research & Training Centre, Gorewada, Nagpur, which were collected during cleaning of cells of captive tiger and leopard, as well as from the surrounding areas of these pens where suspicious scat of free roaming wild carnivore was suspected at Wildlife Research & Training Centre, Gorewada, Nagpur . These samples were processed at WRTC, Nagpur & Dept. of Veterinary Microbiology and Animal Biotechnology Teaching & Research Cells, Nagpur Veterinary College, Nagpur. The details of the sample given in following table no. 1

Table No. 1. : Details of scat samples used in the study.

Sr. No	WRTC code	Lab No.	Sr. No	WRTC code	Lab No.
1	Confiscated sample	F1	13	Panthera 1/5	F13
2	Confiscated sample	F2	14	Panthera 1/3	F14
3	Confiscated sample	F3	15	Panthera ¼	F15
4	Panthera 2/4	F4	16	Panthera 3/2	F16
5	Panthera 2/5	F5	17	PantheraM	F17
6	Panthera 2/1	F6	18	PantheraF	F18
7	Panthera ½	F7	19	PantheraF	F19
8	Panthera 3/3	F8	20	PantheraF	F20
9	Panthera 3/5	F9	21	Tiger Cell No. 01	T1
10	Panthera 1/1	F10	22	Tiger Cell No. 01	T2
11	Panthera ¾	F11	23	Tiger Cell No. 04	T3
12	Panthera 3/1	F12	24	Tiger Cell No. 02	T4

3.1.3 Chemicals, Reagents, Plastic wares, Glasswares and Kits.

Chemicals, reagents and kits used for molecular detection in present study were obtained from Qiagen (Germany), Sigma (USA), Invitrogen (USA), Applied Biosystem (USA) while, plastic wares from Axygen and glassware's from Borosil (India). The different buffers & solution formulations used in present study are given in appendix.

3.1.4 The Oligonucleotide primers

The oligonucleotide primers used for conventional PCR to amplify Cyt. b gene of *Panthera pardus* and *Panthera tigris* were synthesized from Integrated DNA technologies (Eurofins, India; IDT, USA).

3.1.5 Scientific Equipment's

Various scientific equipments available at Department of Veterinary Microbiology and Animal Biotechnology viz., Autoclave (MC Dalal, India), Hot air

oven (MC Dalal, India), ULT Freezer (-80^0 C) (Thermo Electron Corporation, India), Deep Freezer (BlueStar, India), Laminar Flow (Microfilt India), Micropipettes (Eppendorf and GENpet, USA), Refrigerated Centrifuge machine (Thermo Scientific, USA), Thermocycler EppendorfproS (Eppedorf, Germany), Electrophoresis System (Himedia Laboratory Pvt Ltd., India), Transiluminator ((Himedia Laboratory Pvt Ltd., India), Gel Documentation System (ZENITH, Biozene, Agra India).

3.2 METHODOLOGY

3.2.1 Primers

The oligonucleotide primers used for conventional PCR to amplify Cyt. b gene of *Panthera pardus* and *Panthera tigris* are given in Table no. 2.

Table No. 2: List of oligonucleotide primers used for PCR

Primer	Sequence (5'---3')	Reference
Primers of cyt. b of *Panthera pardus*		
Ppo-CbF	GTA AAT TAT GGC TGA ATT ATC CGG	Sugimoto *et al.,* (2006)
Ppo-CbR	CAT AAC CGT GAA CAA TAA TAC GAC	
Primers of cyt. b of *Panthera tigris*		
Pta-CbF	TTT GGC TCC TTA CTA GGG GTG	Sugimoto *et al.,* (2006)
Pta-CbR	CCG TAA ACA ATA GCA CAA TCC CGA TA	

3.2.2 Extraction of Genomic DNA

The Genomic DNA from twenty four fecal samples were extracted by QIAmp mini Stool kit (Cat no. 51504, Qiagen, USA),

1. Weighed 220 mg of scat sample then added 1.6 ml of Buffer ASL to each sample in 2 ml micro centrifuge tube. Vortex continuously till the samples was thoroughly homogenized.
2. Sample was centrifuged at 14500 rpm for 1 min to pellet stool particles, then 1.4 ml of supernatant was collected into a new 2 ml micro centrifuge tube whereas, sediment was discarded.

3. One InhibitEX tablet was added in collected supernatant and immediately vortex it for 1 min or until the tablet is completely suspended. The suspension was kept at room temperature (15–25°C) for 1 min to allow inhibitors to adsorb the InhibitEX matrix.
4. The sample was centrifuged at full speed for 3 min to pellet stool particles and inhibitors bound to InhibitEX matrix.
5. Immediately after centrifugation supernatant were collected into another labelled 1.5 ml microcentrifuge tube, centrifuged at full speed for 3 mins.
6. Added 25µl of proteinase K into a new 2 ml microcentrifuge tube.
7. Collected 600µl supernatant from step 7 to the 2 ml microcentrifuge tube containing proteinase K.
8. Added 600µl Buffer AL and vortex it for 15 s. Incubated at 70°C for 10 min.
9. Added 600µl of ethanol (96–100%) to the lysate, mixed by vortexing.
10. Labeling was done to the lid of a new QIAamp spin column provided in a 2 ml collection tube. 600µl lysate were added into the QIAamp spin column without moistening the rim. Closed the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in new 2 ml collection tube, and discarded the tube containing the filtrate.
11. Added second aliquot of 600µl lysate and centrifuged at full speed for 1 min. the QIAamp spin column placed in a new 2 ml collection tube, discarded the tube containing the filtrate.
12. Repeated step 13 to load the third aliquot of the lysate onto the spin column.
13. Added 500µl Buffer AW1, centrifuged at full speed for 1 min. The QIAamp spin column placed in a new 2 ml collection tube, discarded the collection tube containing the filtrate.
14. Added 500µl Buffer AW2, centrifuged at full speed for 3 min. Discarded the collection tube containing the filtrate.
15. Transferred the QIAamp spin column into a new, labeled 1.5 ml microcentrifuge tube, added 50*µl* AE Buffer and -70^{0}C.

3.2.3 Quantification of DNA

The quantification of extracted DNA was determined by using NanoDrop spectrophotometer (Thermo scientific, USA) instrument. The concentration and optical density was recorded with ratio 260/280 and 230/280.

Initialize the pedal of Nanodrop by milli-Q water then blanked with AE buffer (elution buffer in QiaAmp DNA Mini Stool Kit) and then 1 µl of DNA was used for quantification (Thermo scientific, NANODROP 1000 Spectrophotometer).

3.2.4 Ampification of cytochrome b

The cytochrome b gene were amplified by using species specific primers 10 picomole each (Pta-cy b F, Pta-cy b R; Ppo-Cy b F, Ppo-Cy b R). The PCR reaction was set as follows,

Component	**Volume (µl)/Reaction**
DNA	3
10X PCR Buffer	5
25mM MgCl2	6
10 mMdNTPs	1.5
Pta-Cy b F	2
Pta-Cy b R	2
Ppo-Cy b F	2
Ppo-Cy b R	2
Taq polymerase (1U/ µl)	1.5
NFW	25
Total volume	50

Cyclic conditions for amplification of Cytochrome b gene,

Step	Temperature	Time	
Initial denaturation	94^0C	5 mins	
Denaturation	94^0C	30 sec	45 cycles
Annealing	60^0C	30 sec	
Extension	72^0C	45 sec	
Final extension	72^0C	10 mins	
End of the PCR cycles	4^0C	∞	

3.2.5 Confirmation of Cytochrome b gene amplicon by agarose gel electrophoresis

The PCR products were determined by agarose gel electrophoresis (AGE) in 3% agarose gel containing 0.5 µg/ml ethidium bromide (Sigma) in 0.5 X tris borate EDTA (TBE) buffer. 10 µl of PCR products was mixed with 2 µl of 6X loading dye (bromophenol blue) and loaded into wells of prepared gel. The 50 bp DNA ladder (#SM0371, Thermo Scientific, USA) was loaded with PCR products to check the specific band size. Electrophoresis was carried out at 60 V of gel in 0.5 X TBE running buffer in submarine electrophoresis System till the bromophenol blue (6X loading dye) reached 2/3rd of the gel. The gel was visualized using the transluminator and photographed in gel documentation system.

The two 156 bp and one 271 bp PCR products were run in 1.5% AGE in TAE buffer with 50bp DNA ladder and the amplicons were cut precisely for gel purification. Gel purification was carried out by QIAquick® gel extraction kit (Cat. # 28704).

3.3.6 Gel extraction Protocol

1. DNA fragment was excised from the agarose gel with clean, sharp scalpel.
2. Gel slice was weighted in a colorless tube micro prelabeled microcentrfuge tube.

3. The three volume of Buffer QG to one volume of gel in this proportion viz. 100 mg gel= 100µl was considered for quantity adjustment.
4. Incubated at 50^0C for 10 minutes, the content was vortexd intermittently in every 2-3 minutes.
5. One gel volume of isopropanol was added to the sample mix.
6. QIAquick spin column was placed a in a provided 2ml collection tube. To bind DNA, sample was added to the QIAquick column and centrifuged for 1 min until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volume of > 800µl. Load and spin again.
7. As DNA will be subsequently be used for sequencing, 500 µl Buffer QG was added to the QIAquick column and centrifuged for 1 min The flow through was discarded and the QIAquick column was placed back into the same tube.
8. To wash, 50 µl Buffer PE was added to QIAquick column and centrifuged for 1 min. The flow-through was discarded and the QIAquick column was placed back into the same tube.
9. QIAquick column was centrifuged in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
10. QIAquick column was placed into a clean 1.5 ml micro centrifuge tube.
11. DNA was eluted by adding 50 µl Buffer EB (10 mMtris-HCL, pH-8.5) or water to the center of the QIAquick membrane and centrifuged the column for 1 min. For increased DNA concentration added 30 µl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuged for 1 min.
12. Purified DNA was analysed on gel
13. The gel eluted products were quantified at OD 230/260 260/280 using Nano drop spectrophotometer.

3.2.7 Sequencing

Two PCR amplicons of size 156 bp and One of 271 bp, were sent for commercial sequencing from Eurofins, Bangaluru, India.

3.2.8 Sequence and Phylogenetic analysis of Cytochrome b gene

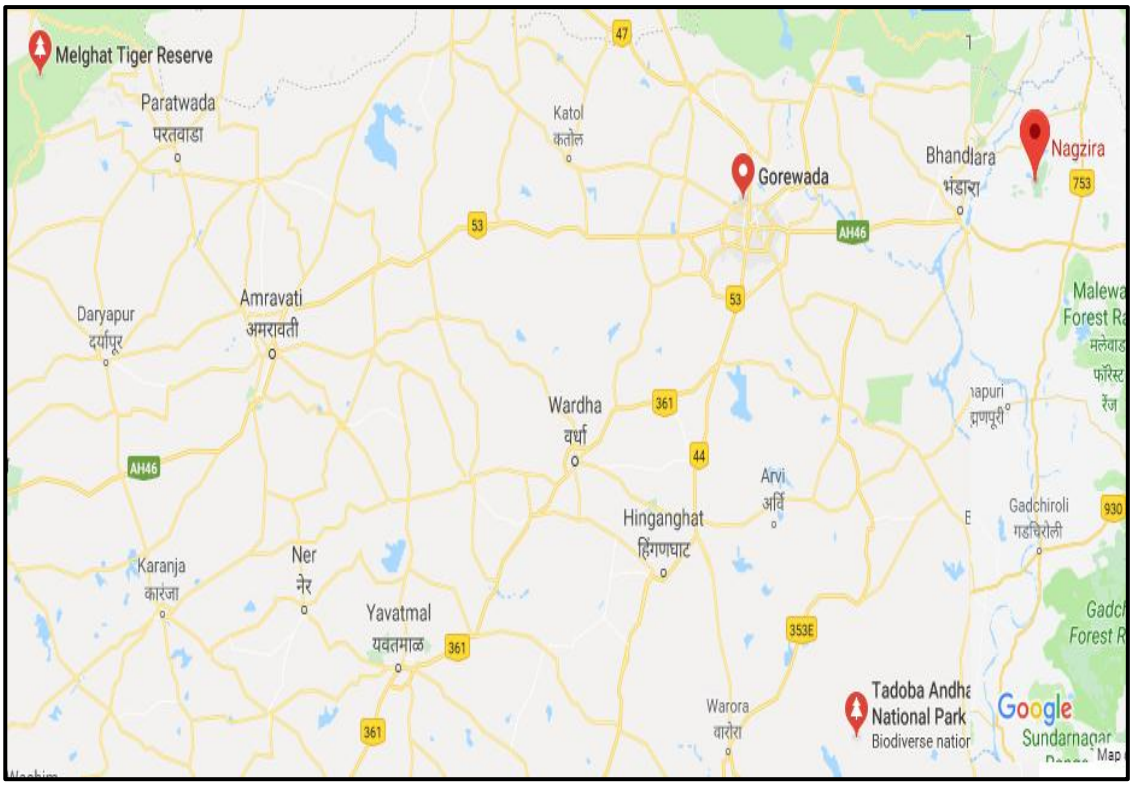
The cytochrome b sequences were received after sequencing, were processed for base call and trimmed using Chromas software (Version 2.1.8). The clean trimmed sequences were processed for homology sequences using BLASTn (Nucleotide BLAST) online interface of GenBank online (https://blast.ncbi.nlm.nih.gov/Blast) and mismatch sequences were reanalyzed by Chromas for base call. Reverse sequences were reverse complemented using Chromas. The contig sequences were prepared by using online software and consensus sequences were saved for afterward analysis using BLASTn program. The cytochrome b sequences of *Panthera pardus* and *Panthera tigris* in present study (PF1_Ppardus_NGP_MS_IND; PF12_Ppardus_NGP_MS_IND; T3_Ptigris_NGP_MS_IND) with different cytochrome b nucleotide sequences (EF056507, KX655614, KP001507, AB817079, AB817078, AB211401, JN709941, EF063721, KP202265, AB211402, KJ850246, AB211407, KJ866876, JF720092, EF551002, EF199742, KJ545526, KF297576, KC879291, JQ040937, JQ040930, JQ040917, JQ040913, HM185182, JF357973, JF357972, EF179375, AB211408, AF053034, JF720140, JF720129) available on NCBI were taken and trimmed using Chromas software (Version 2.1.8) for multiple sequence alignment. Some control animal sequences Felis californica (AJ300702), *Neofelis diardi* (HM748847), *Neofelis diardi* (HM748855), *Neofelis nebulosa* (HM748842), *Canis aureus* (KT343786), *Bubalis bubalis* (EU908278*), Capra hircus* (FJ785333), *Canis lupus* (KJ162939), *Puma concolor* (EF373820), *Felis catus* (KM224380), *Prionalurious bengalensis* (AJ300700) which were not trimmed as they don't match with our sequences. All 44 sequences were analyzed multiple alignment was done using *Clustal W* embedded in the MEGA7 software. The aligned data was used for phylogenetic analysis in MEGA 7 software's and the evolutionary analysis was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model. The initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated.

RESULTS AND DISCUSSION

India has rich wildlife heritage, it has long history and tradition of wildlife conservation. It maintains balance of nature by regulation of population of different species by self-regulation and feedback, passage of food and energy through series of food chain, terrestrial and aquatic animals and helps the environment for self-sustaining system. But because of gradual emergence of humans being a major evolutionary force, peoples are exploiting the wildlife by illegal vocations viz., hunting, destruction of wildlife habitats, deforestation etc. because of these manmade unnatural events the living chain gets disturbed and wild animals comes in human territory, where incidences like human wildlife conflicts leading to loss of life. Due to social and bio geographical characteristics, developing regions of world viz., South and Southeast Asia are more prone for human wildlife conflict (HWC). Sometimes conflict happens somewhere in forest and information about is scanty, so to decipher the involvement of species. The molecular diagnostic techniques found to be of gold standard in such cases. In India, the HWC case studies were conducted over past few decades, which showed that 90% of country is affected by HWC besides other forestry issues (Shaurabh *et al.,* 2017). Most of the time, identification of species involved is of prime importance, but getting samples is always a trickiest part, so investigator have tried several approaches, one of them is scat samples. The nuclear DNA vs mtDNA has to be choose to identify the species. Mitochondrial DNA for investigation found to be of choice owing to several reasons like mtDNA is more stable than nuclear DNA (nDNA) and have more number of DNA copies in comparison with nuclear DNA. It can be analyzed even from the highly damaged, degraded or very small quantity of the samples (Burgener *et al.,* 1998).

Vidhrabha region is most forest covered area, interconnected tiger reserves as well as its connectivity to forest of Madhya Pradesh and presence of large number of wildlife population especially tigers & panthers lead to several incidences of HCW. There is need of molecular approach to identify the species as molecular forensic tool. So present study was planned to identify *Panthera pardus* and *Panthera tigris* by non-invasive fecal samples based on amplification of mitochondrial gene cytochrome b gene and its sequencing.

Figure 1: Distribution of Wildlife sanctuaries, reservoirs Centre's in Vidarbha.



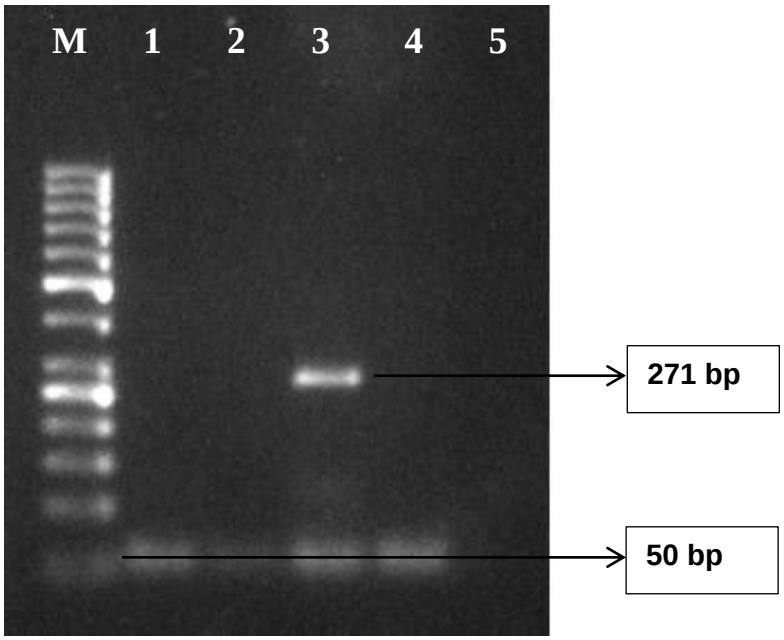
As per MOU, between FDCM, Ltd, Nagpur and MAFSU, Nagpur, the non-invasive collected fecal samples (scat) were received from Wildlife Research & Training Centre (WRTC), Gorewada, Nagpur. It's always difficult to identify species on basis of fecal morphology. Scat samples are one of the best easily available sample in captivity as well as can be collected in wild also. The fecal samples (scat) sample has been proven for molecular species identification (Sugimoto *et al.,* 2006; Karmacharya *et al.,* 2016).

The extraction of good quality DNA is an important part in forensics approach, so isolation of genomic DNA including mtDNA was done by using QIAamp DNA stool mini kit as per manufactures instructions. In another study to estimate tiger population by non-invasive fecal samples for designing of conservation measures, DNA extraction was carried out by different methods including QIAamp DNA stool mini kit to analyze the best one. The study revealed, QIAamp DNA stool mini kit was good method for DNA extraction over conventional methods (Bhagavtula *et al.,* 2006). The same kit have been used by the several researchers (Sugimoto *et al.,* 2006; Karmacharya *et al.,* 2016).

The quantification of extracted DNA was done by using NanoDrop spectrophotometer. The concentration and optical density was recorded with 260/280 and 230/280 ratio. The fecal samples showed DNA with high DNA purity with 1.8 to 1.9 OD, as the impurity of DNA is one of the factor for false negative results in PCR.

The extracted DNA samples were processed for multiplex PCR using species specific published primers as, *Panthera pardus* specific (Ppo-CbF/Ppo-CbR) and *Panther tigris* specific (Pta-CbF / Pta-CbR) primers (Sugimoto *et al.,* 2006). All the scat samples (24 Nos.) were analyzed by multiplex PCR using tiger & leopard species primers. All the amplicons were confirmed by their expected size by agarose gel electrophoresis keeping DNA ladder for size confirmation. Amongst 24 scat samples tested, 19 samples revealed as expected leopard specific 156 bp and one sample showed a tiger specific amplicons of 271bp. The representative image of the amplification is depicted in the Fig 2 & 3. The same primers have been previously used by Sugimoto *et al.,* (2006), where they have shown a 156bp amplicons for *Panthera pardus* and 271bp for the *Panthera tigris.* Our results are in accordance with this report.

Figure 2: Agarose gel electrophoresis showing Cytochrome b gene of *Panthera tigris* (271 bp) by Multiplex PCR of fecal DNA.



M - DNA Marker (50 bp)

Lane 1- T1

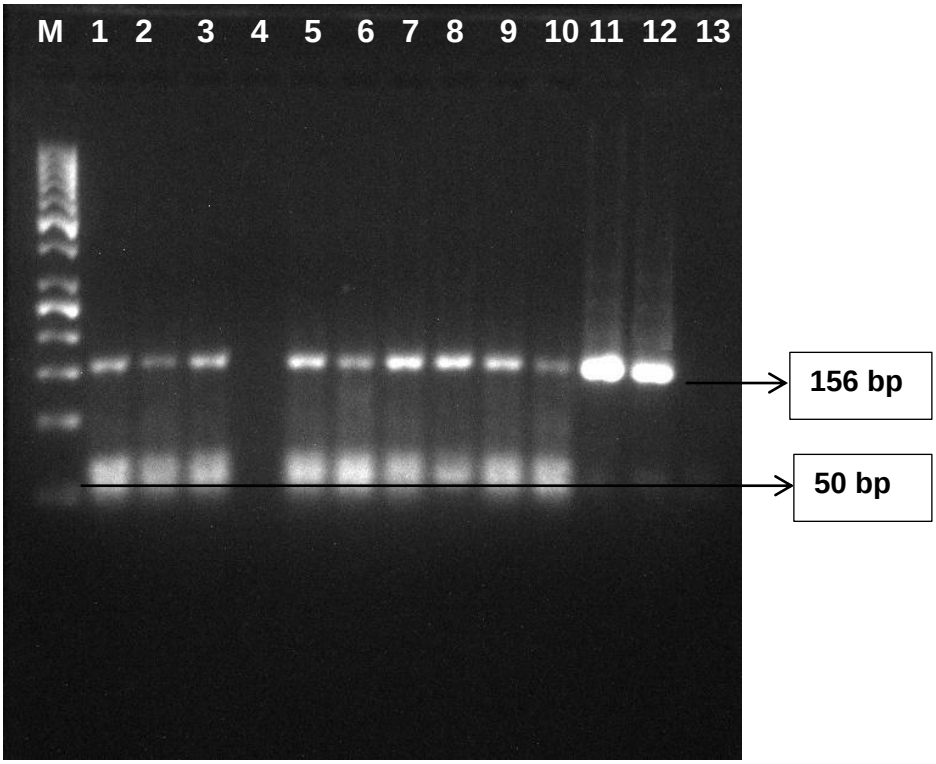
Lane 2- T2

Lane 3- T3

Lane 4- T4

Lane 5- NTC

Figure 3 : Agarose gel electrophoresis showing Cytochrome b gene of *Panthera pardus* (156 bp) by Multiplex PCR of fecal DNA.



M- 50 bp DNA ladder
Lane 1- F1
Lane 2- F2
Lane 3- F3
Lane 4- F4
Lane 5- F5
Lane 6- F6
Lane 07- F7
Lane 08- F8
Lane 09- F9
Lane 10- F10
Lane 11- F11
Lane 12- F12
Lane 13- NTC

The use of scat samples have been successfully employed to identify the Bengal tiger and Snow leopard species on 420 fecal samples revealed that cytochrome b gene PCR using tiger specific primers (TIF/TIR) and leopard specific primers (CYTB-SCT-PUN-F/ CYTB-SCT-PUN-R) could amplified 162 bp and 150 bp products indicating 62 % samples identified as tiger with 162 bp size and out of 31 samples, 65% samples were identified as snow leopard with amplicon of 150 bp size.

The further analysis showed that 68 samples were not identified because of poor DNA quality and also 26% field misidentification of target species in tigers samples and 28% field misidentification of target species in leopard samples (Mukherjee *et al.,* 2007). The Cyt b gene was also used for species identification for illegal trades (Tsai *et al.,* 2007) also in confiscated species in zoo visit of free roaming carnivore confirming the visit of the leopard (Verma *et al.,* 2003) . In our study we have also received (3) confiscated scat samples from surrounding of captivity of leopard at WRTC, Gorewada, Nagpur. On further analysis by cytb gene amplicons indicated the 156bp product so we confirmed it as an leopard visit, which was further confirmed by the sequencing of one sample. Hence our present study showed that the specific amplification of cytb gene for leopard & tiger in any type of scat samples. The predatory animal identification can also be done using cytb gene as evident by a study indicating predation behavior of sympatric jaguar and puma, showed 146 bp fragment PCR & sequencing of amplified products reveals the detection of Domestic dog (C*anis familaris)*, Wild boar (*Sus scrofa*) and ocelot (Farrell *et al.,* 2000). But in our study, we could substantiate the only tiger & leopard species identification as primers are being designed for these species which was further confirmed by the sequencing data.

For validation, the PCR amplicons regarding their species identification, PCR products were further processed for sequencing using gel purified PCR amplicons to confirm the specificity of primers towards target species of leopard and tiger. The sequences data obtained after commercially sequencing was processed for base call and trimmed using Chromas software (Version 2.1.8). The clean trimmed sequences were processed for homology sequences using BLASTn online interface of GenBank online (http:/blast.ncbi.nlm.nih.gov/) and mismatch sequences were reanalyzed by Chromas software for base call.

Figure 4 : Molecular Phylogenetic analysis of partial cytochrome b sequence of *Panthera pardus* and *Panthera tigris* of Wildlife Research and Training Centre, Gorewada, Nagpur with different species by Maximum likelihood method based on Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches, the tree is drawn to scale, with brach length measured in the number of substitutions per site. All the analysis was carried out using MEGA 7.

Reverse sequences were reverse complemented using Chromas software. The contig sequences were prepared by using online software and consensus sequences were analyzed by BLASTn program. The cytochrome b sequences of *Panthera pardus* and *Panthera tigris* in present study (PF1_Ppardus_NGP_MS_IND; PF12_Ppardus_NGP_MS_IND and T3_Ptigiris_NGP_MS_IND) were analyzed by BLASTn. The percent homology of *Panthera pardus* sequences showed 99 to 97 % identity to *Panthera pardus* spp. The *Panthera tigris* sequences showed 99 % identity with *Panthera tigris* spp. PF1_Ppardus_NGP_MS_IND showed 99 % homology with *Panthera pardus*, Maharashtra (EF056507.1), PF12_Ppardus_NGP_MS_IND showed 99 % homology with *Panthera pardus orientalis*, China (KX655614.1) and T3_Ptigiris_NGP_MS_IND showed 99 % homology with *Panthera tigris altica*, Bangkok, China (KF297576). The BLASTn homology data is depicted in table no. 3; 4 and 5. This indicated that *Panthera pardus* cyt b gene sequences under study were closely similar to *Panthera pardus* spp. genomic cytb from Maharashtra (India), Beijing (China), Netherland, Japan. The *Panthera tigris* cytochrome b gene sequence from the present study revealed that close similarity with cyt b mtDNA genome of the *Panthera tigirs* spp. from China, Netherland, USA, Japan. All the sequence under study were matching with their respective panther spp. viz. *Panthera pardus* & *Panthera tigris* indicating the amplicons of cytb gene of respective spp.

Table No. 3: Sequences producing significant alignments of PF1_*Ppardus*_NGP_MS_IND

Description	Max score	Total score	Ouery cover	E value	Ident	Accessi NO.
EF056507_Panthera_pardus_Pune_Maharashtra_India	293	293	100%	1e-75	99	EF056507
KX655614_Panthera_pardus_orientalis_Beijing_China	288	288	100%	5e-74	98	KX655614
KP001507_Panthera_pardus_Zuid_Holland_Netherlands	288	288	100%	5e-74	98	KP001507
AB817079_Panthera_pardus_orientalis_Hokkaido_Japan	288	288	100%	5e-74	98	AB817079
AB817078_Panthera_pardus_orientalis_Hokkaido_Japan	288	288	100%	5e-74	98	AB817078
AB211401_Panthera_pardus_orientalis_Hokkaido_Japan	288	288	100%	5e-74	98	AB211401
KP202265_Panthera_pardus_USA	288	288	100%	6e-73	98	KP202265
KJ866876_Panthera_pardus_japonensis_Beijing_China	284	284	100%	6e-73	98	KJ866876

Table No. 4 : Sequences producing significant alignments of PF12_*Ppardus*_NGP_MS_IND

Description	Max score	Total score	Ouery cover	E value	Ident	Accessi NO.
KX655614_Panthera_pardus_orientalis_Beijing_China	273	273	100%	1e-69	99	KX655614
KP001507_Panthera_pardus_Zuid_Holland_Netherlands	273	273	100%	1e-69	99	KP001507
AB817079_Panthera_pardus_orientalis_Hokkaido_Japan	273	273	100%	1e-69	99	AB817079
AB817078_Panthera_pardus_orientalis_Hokkaido_Japan	273	273	100%	1e-69	99	AB817078
JN709941_Panthera_pardus_Hanoi_Vietnam_Baohoamai	273	273	100%	1e-69	99	JN709941
EF056507_Panthera_pardus_Pune_Maharashtra_India	273	273	100%	1e-69	99	EF056507
AB211407_Panthera_pardus_Orientalis_Hokkaido_Japan	273	273	100%	1e-69	99	AB211407
AB211401_Panthera_pardus_Orientalis_Hokkaido_Japan	273	273	100%	1e-69	99	AB211401

Table No. 5 : Sequences producing significant alignments of F3_*Ptigris*_NGP_MS_IND

Description	Max score	Total score	Ouery cover	E value	Ident	AccessiNo.
KF297576_Panthera_tigris_Bangkok_Thialand	477	477	100%	9e-131	99	KF297576
KC879291_Panthera_tigris_Bangkok_Thialand	477	477	100%	9e-131	99	KC879291
JQ040937_Panthera_tigris_altica_NewYork_USA	477	477	100%	9e-131	99	JQ040937
JQ040930_Panthera_tigris_altica_NewYork_USA	477	477	100%	9e-131	99	JQ040930
JQ040917_Panthera_tigris_altica_NewYork_USA	477	477	100%	9e-131	99	JQ040917
JQ040913_Panthera_tigris_altica_NewYork_USA	477	477	100%	9e-131	99	JQ040913
HM185182_Panthera_tigris_altaica_Daegu_Korea	477	477	100%	9e-131	99	HM185182
JF357973_Panthera_tigris_altaica_AdelaideSA_Australia	477	477	100%	9e-131	99	JF357973
JF357972_Panthera_tigris_corbetti_AdelaideSA_Australia	477	477	100%	9e-131	99	JF357972
EF179375_Panthera_tigris_jacksoni_Pahang_Malaysia	477	477	100%	9e-131	99	EF179375
AB211408_Panthera_tigris_altica_Hokkaido_Japan	477	477	100%	9e-131	99	AB211408
AF053034_Panthera_tigris_altica_NewYork_USA	477	477	100%	9e-131	99	AF053034

The 44 sequences including three sequences of present study were analyzed using the Clustal W embedded program in the MEGA 7. Afterward the aligned data was used for phylogenetic analysis MEGA 7 software's and the evolutionary analysis was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-635.52) was obtained. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The phylogenetic tree showed typical distributions of Leopard and tiger species. The *Panthera pardus* and related subspecies were clubbed together into a single clade. The *Panthera pardus* cytochrome b sequences of *Panthera pardus* PF1_Ppardus_NGP_MS_IND leopard was found closely associated with *Panthera pardus* from Westerncape, South Africa and PF12_Ppardus_NGP_MS_IND was found *Panthera pardus* origin Anhui, China and are being in Clad I (fig. 04). Hence, from such differential cladding the geographical distribution of *Panthera* spp. could not be concluded out. However placing of these leopards under study in the *Panthera pardus* group signifies these belong to the *panthera pardus* species only.

The T3_Ptigris_NGP_MS_IND tiger cytochrome b sequence of present study based phylogenetic analysis revealed that all the tiger specific sequences were placed in species specific clad II whereas the other species are placed in different groups (fig. 04). The tiger (T3_Ptigiris_NGP_MS_IND) from the present study was found closely related to *Panthera tigris altica* from SA, Australia.The phylogenetic analysis shown that all the species were placed in their species specific groups. So sequence analysis and further phylogenetic analysis is validation of their species. Our result also in accordance with research who has evaluated the cyt b gene for species identification by amplicons sequencing (Masuda *et al.,* 1995; Farrell *et al.,* 2000; Awad *et al.,* 2015). The molecular phylogenetic relationship amongst different species in *Felidae* family conducted on cytochrome b gene yielded average pair-wise similarity values between felids ranging from 94 to 99% and 85 to 99%, respectively (Masuda *et al.,* 1995). Similarly, sequencing of cyt b gene reveals the detection of domestic dog (*Canis familaris)*, wild boar (*Sus scrofa*) and ocelot as predation animal of small carnivores (Farrell *et al.,* 2000). The cytochrome b partial sequencing and its

analysis found to be useful for detection of 19 species including wild & domestic animals. (Hsieh *et al.,* 2001) as well some avian species like Chicken (*Gallus gallus*), Muscovy duck (*Cairina moschata*), Japanese quail (*Coturnix japonica*), Laughing dove (*Streptopelia senegalensis*), and Rock pigeon (*Columba livia*) (Awad *et al.,* 2015), 44 different animal species covering the 5 major vertebrate groups (i.e. mammals, birds, reptiles, amphibians and fishes) were also identified (Parson *et al.,* 2017). Cytochrome b gene sequencing has also reported in detection of divergence of 2 lineages of earlier identified squirrel app. 1.32 MYA, which further helped in identification of new species viz., Humboldt's Flying squirrel (Arbogast *et al.,* 2017). Similarly for hybridization effect of translocation of boars in Argentina to acesss the origin of wild domestic population of boars the cytrochrome b was found suitable for species specific identification (Sagua *et al.,* 2018).

Hence, from the sequencing of amplicons of cyt b gene we can conclude that cytochrome b gene has been robust for identification of species.

The present study concludes that, scat sample, a very easily obtained sample in wild as well as captivity, non-invasive method, confiscated scats samples etc. can be employed for the detection of tiger & leopard species by cytb amplification using the specific primers under study. The present study also delineate that using these multiplex PCR approach for amplification of cytb of tiger & leopards and its sequencing can be a tool for forensic cases, for monitoring of the movement of animals, trades of animals and its products, HWC cases, endangered species identification etc. and also its concludes the scat sample mtDNA genetic analysis is robust for identification of species

SUMMARY AND CONCLUSION

Wildlife comprises variable types of wild animals in different families, it's one of the cultural beauty in Asia. It's the topic of prestige and pride for India who harbors such diversified wildlife population, but their population monitoring is difficult as they live in dense forest areas an sometime comes in human vicinities. The conflicts between humans and animals are getting increasing day by day due to increased industrialization, deforestation, wildlife trades, poaching etc. In such conditions the establishment of animal species involvement remains one of trickest part, as every time you did not get live sample like blood, tissues , bones etc. The molecular forensic science need a DNA to identify the species. In such cases the scat samples which can be obtained at crime scene or nearby, or in wild or in captivity which is one of humane, easily available sample from wild animal that can be explored for species identification. The scat samples has also some difficulties in case of carnivore to differentiate the predatory animals, so identification of target species based on the suitable marker and species specific gene is very important. The mitochondrial DNA was found to be of choice owing to several reasons like mtDNA is more stable than nuclear DNA (nDNA) and have no. of DNA copies in comparison with nuclear DNA. It can be analyzed even from the highly damaged, degraded or very small quantity of the samples.

In vidharbha region, which is highly blessed with flora and fauna, the establishment of species in various incidences required and found essential species as molecular forensic tool. Hence, the present study was planned to decipher the possibility of scat (fecal samples) to establish the species identification for *Panthera pardus* and *Panthera tigris* by using mtDNA cytochrome b gene bases amplification and validate it by sequencing and its analysis.

The coded scat samples (24) including a confiscated scat samples nearby the leopard cells, were received from the Wildlife Research and Training Centre, Gorewada Nagpur. The all samples (24) were processed for DNA isolation using QIAamp mini stool DNA kit followed by its concentration & purity checking by Nanodrop spectrophotometer. The samples were analyzed with species specific primers targeted for Cytochrome b gene of *Panthera pardus* and *Panthera tigris* using multiplex PCR. The two amplicons including one confiscated scat sample amplicons & one amplicons from tiger were

commercially sequenced. The raw sequenced sequence data were further analyzed using different bioinformatics software tools viz., Chromas software (Version 2.1.8), Clustal W, BioEdit, BLASTn and phylogenetic analysis was done using maximum likelihood by MEGA 7 software.

The PCR analysis revealed expected amplicon size 156 bpfor 19 samples out of 20 *Panthera pardus* scat samples and271bp in only one sample out of four *Panther tigris* scat samples. This indicated that PCR amplicons was identified with specific species of *Panthera pardus* and *Panthera tigris* and matched as per the data of wildlife kept at WRTC, Gorewada. We could also identify the confiscated sample as an of free roaming panther pardus (leopard) who is visiting the leopards kept in captivity at WRTC, Gorewada, Nagpur. The cytochrome b sequences of *Panthera pardus* and *Panthera tigris* in present study (PF1_Ppardus_NGP_MS_IND; PF12_ Ppardus_NGP_MS_INDand T3_Ptigiris_NGP_MS_IND) were analyzed by BLASTn showed showed 99 to 97 % identity for *Panthera pardus*. PF1_Ppardus_NGP_MS_IND showed 99 % homology with Panthera pardus, Maharashtra (EF056507.1), PF12_Ppardus_NGP_MS_IND showed 99 % homology with Panthera pardus orientalis, China (KX655614.1) and T3_Ptigiris_NGP_MS_IND showed 99 % homology with Panthera tigris altica, China (KF297576).

In our study the phylogenetic tree showed a typical distributions of *Panthera pardus* and *Panthera tigris* species. The *Panthera pardus* and related subspecies were clubbed together into a single clade. The cytochrome b sequences of *Panthera pardus* PF1_Ppardus_NGP_MS_IND leopard was found closely associated with leopard from the Westerncape, South Africa and PF12_Ppardus_NGP_MS_IND was found *Panthera pardus* origin Anhui, China and tiger (T3_Ptigiris_NGP_MS_IND) of the present study was found closely related to *Panthera tigris altica* from SA, Australia. Hence, because of such different cladding pattern, the geographical distribution of Panthera spp. could not be concluded out.

The present study concludes that, scat sample, a very easily obtained sample in wild as well as captivity, non-invasive method, confiscated scats samples etc can be employed for the detection of tiger & leopard species by cytb amplification using the specific primers under study. The present study also delineate that using these multiplex PCR approach for amplification of cytb of

tiger & leopards and its sequencing can be a tool for forensic cases, for monitoring of the movement of animals, trades of animals and its products, HWC cases, endangered species identification etc. and also its concludes the scat sample mtDNA genetic analysis is robust for identification of species.

Further studies should carried out to established DNA fingerprint of maximum wildlife animals in sensitive areas of wildlife's associate with HWC also established phylogenetics of Indian tigers and leopards should be done by collecting samples throughout India to search for breeding in natures and associated issues.

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APPENDIX

1. Removal of DNase contamination

a) Glassware

Properly cleaned glasswares were filled with 0.1 % of DEPC water and allow it to stand for overnight (12 hrs) at 37 ^{0}C, and then heated to 100 ^{0}C for 15 mins to remove remnants of DEPC.

b) Plasticware

Plastic ware not resistant to chloroform were thoroughly rinsed with 0.1M NaOH, 1mM EDTA followed by DEPC treated water, while chloroform –resistant plastic ware were rinsed with chloroform to inactivate DNases.

c) Water

1 ml of Diethyl pyrocarbonate (DEPC) was added into 999 ml of tripple distilled water and shaken vigorously to mix DEPC into water and it was incubated for 12 hrs at 37 ^{0}C. then autoclaved to remove minor traces of DEPC.

2. Reagents used for DNA extraction

QiaAmp DNA mini stool kit comprises of different buffers as,

a. Buffer ASl
b. inhibitEX tablet
c. Proteinase k
d. Buffer AL
e. Absolute ethanol (96-100)
f. Buffer AW1
g. Buffer AW2
h. Buffer AE

3. Reagents for Agarose Gel electrophoresis

i) Tris Borate EDTA (TBE) buffer (5X)

Stock solution:

Tris base	54 gm
Boric acid	27.5 gm
EDTA acid	20 ml
H2O	1000 ml

Working concentration of TBE buffer (0.5 X)

TBE buffer	50 ml
H2O	450 ml

ii) Tris- Acetate (TAE) Buffer (5X Stock solution)

Tris base/Tris buffer	242.0
Glacial acetic acid	57.1
0.5 M EDTA (PH 8.0)	100 ml

Storeat room temperature

For use dilute 1:10 with water for agarose gel electrophoresis

4. 0.5 M EDTA (pH- 8.0)

EDTA	18.61 gm
H2O	100 ml

(Adjust the pH to 8.0 with 1.0 N NaOH)

Adjust the volume of the solution to 100 ml with double distilled miliQ water H2O.

Autoclave and store at room temperature.

5. Ethidium bromide solution (10mg/ml)

Ethidium bromide	0.1gm
Distilled water	10.0 ml

6. Gel loading dye

Bromophenol blue	0.25%
Sucrose in water	40%
Distilled water	100ml

Stored at 4˚c

VITAE

The author **POOJA SANJAY ACHARYA** was born on 5th March 1994 at Ambejogai, district Beed, Maharashtra. She complited her Higher education in 2011 from Yashwantaro Chavan Mahavidyalaya, Ambejogai. She did her undergraduation from College of Veterinary and Animal Sciences, Udgir. During her college days she participated in 7th international Clinical case Conference held at Nammakal, Chennai. During her PG academic she attended National Conference IAVMI, 2017 held at Nagpur and VIBCON, 2017. She got ISVIB-VC & RI Namakkal Silver Jublee Student Travel Grant Award 2017 in VIBCON, 2017.

Date:-

Place:-

Signature

(Pooja Sanjay Acharya)

THESIS ABSTRACT

a)	Title of the thesis	:	**GENETIC ANALYSIS OF PANTHERA PARDUS AND PANTHERA TIGRIS BY mtDNA PCR**
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ABSTRACT

Vidarbha region, is blessed with flora & fauna where several cat families members are being residing in different forest. However, none of the report signifies the genetic analysis of these wild animals. There are always man and

animal conflict reported mentioning animals being man eater or so. So the presence of particular animal is to be firmly confirmed to take any further action to avoid the killing of innocent one. Similarly, the wild animal sample collection remains one of trickiest part, however the scat sample can easily obtained by humane methods in captivity as well as in wild. With advances of genetic techniques using the scat samples to identify the species need to evaluate to enforce the protection of endangered species. Hence, the present study was undertaken to develop a rapid and cost-effective protocol for the reliable identification of tigers and leopards species from sympatric carnivore scats. The coded scat samples (24) including a confiscated scat samples nearby the leopard cells, were received from the Wildlife Research and Training Centre, Gorewada Nagpur. The samples were processed for DNA isolation using QIAamp DNA mini stool kit followed by its quantification by Nanodrop spectrophotometer. The DNA was analyzed with species specific primers targeted to mtDNA Cytochrome b gene of *Panthera pardus* and *Panthera tigris* using multiplex PCR. Furthermore, two amplicons including one confiscated scat amplicon from leopard and one amplicon from tiger were commercially sequenced. The received raw sequence data were further analyzed using different bioinformatics software tools viz., Chromas software (Version 2.1.8), clustal W, BioEdit, BLASTn and phylogenetic analysis was done using maximum likelihood by MEGA 7 software. The mtDNA analysis using the PCR showed a typical 156 bp amplicons in 19 samples of panthera pardus & 271bp amplicons for one tiger species. The homology search of mtDNA cytb gene partial sequence analysis by BLASTn also revealed our sequences are of *Panthera pardus* and *Panthera tigris* in origin as they showed homology upto 99% with respective species. The phylogenetic analysis also placed the *Panthera pardus* and *panthera tigirs* sequences in their respective species clads. From the present study, we conclude that mtDNA cytochrome b gene amplification with species specifc primers under study can be employed for the detection of *Panthera pardus* and *Panthera tigris* from scat sample successfully. Also scat sample found to useful in the monitoring of species movement, forensics tools for species identification as well in confiscated samples and in human animal conflicts cases.

प्रबंध सारांश

अ)	प्रबंधचे शीर्षक	:	**''पॅन्थेरा पारडस आणि पॅन्थेरा टायग्रिसच्या कलकनुकोशिकेतील डीएनएद्वारा अनुवांशिक विश्लेषण''**
ब)	विद्यार्थ्याचे पूर्ण नाव	:	**आचार्य पूजा संजय**
क)	मार्गदर्शक नाव आणि पत्ता	:	**डॉ. पी. ए. टेंभुर्णे**, सहाय्यक प्राध्यापक, पशुवैद्यकीय सूक्ष्मजीवशास्त्र आणि पशु जैवतंत्रज्ञान विभाग, नागपूर पशुवैद्यकीय महाविद्यालय,
ड)	प्रदान करन्यात येणारी पदवी	:	**स्नातकोत्तर पदवी** (एम.व्ही. एससी)
ई)	मुख्य विषय	:	**पशुजैवतंत्रज्ञान**
फ)	पदवी प्राप्त करण्याचे वर्ष	:	**२०१८**
ग)	प्रबंधमध्येमध्ये एकूण पृष्ठांची संख्या	:	**३२**
ह)	सारांशातील एकूण शब्द	:	**३१९**
इ)	विद्यार्थ्यांची सही	:	
ज)	अग्रेषित करणाऱ्या अधिकार्याची स्वाक्षरी, नाव आणि पत्ता	:	(सहयोगी अधिष्ठाता) नागपूर पशुवैद्यकीय महाविद्यालय, नागपूर

सारांश

विदर्भ क्षेत्र हा विविध प्रकारच्या वनस्पती आणि प्राणिजातींनी समृद्ध आहे. मार्जरकुळातील अनेक प्राणिसदस्य विदर्भातील जंगलात राहतात. तथापी जंगली

जनावरांच्या अनुवांशिक विश्लेषणास सूचित करणारा अहवाल उपलब्ध नाही. नंहमीच मानवी आणि पशुसंघर्षामधे मनुष्य खाणाऱ्या प्राण्याचा उल्लेख झालेला असतो. त्यामुळे चूकून निष्पाप प्राण्याची हत्या टाळण्यासाठी व हल्ल्यापासून बचाव करण्यासाठी कोणत्याही विशिष्ठ कृतीची व त्याच्या उपस्थितीची पुष्ठी निर्वादितपणे केली गेली पाहिजे. वन्यपशु नमुने गोळा करणे सर्वात कठीण काम आहे. तथापि त्याच्या विष्ठेचा नमुना सहजपणे बंदिवासात तसेच जंगलात मानवीय पद्धतीने मिळवता येते. संकटग्रस्त प्रजातींच्या संरक्षणासाठी प्रजातींच्या ओळख पटवण्यासाठी या नमुन्यांच्या उपयोगाद्वारे अनुवांशिक तंत्रज्ञानाच्या प्रगतीने मूल्यांकन करणे आवश्यक आहे. वाघ आणि बिबट्यांच्या विष्टेतुन या प्रजातींचे इतर मांसाहारी प्राण्यापासून विश्वसनीय ओळख करण्यासाठी जलद आणि जास्त प्रभावी पद्धत शोधण्यासाठी सदरील प्रकल्प हाती घेण्यात आला. वन्यजीव संशोधन व प्रशिक्षण केंद्राकडून तेथील वाघ व बिबट यांच्या पिंजऱ्यामधील तसेच सभोवतील परिसरातील विष्ठेचे नमुने (२४) सदरील प्रकल्पासाठी प्राप्त झाले. प्राप्त केलेल्या नमुन्यातून डीएनए अलगाव साठी कॉयअॅम्प डीएनए मीनी स्टूल कीटचा वापर करून काढण्यात आले. त्यानंतर जणूकचे अतिसुक्ष्म थेंब पंक्तिप्रकाशमापक द्वारे मूल्यांकन करण्यात आले. बहुमिश्रित शृंखला साखळीची प्रतिक्रियेचा वापर करून पॅथेरा पारडूस आणि पॅथेरा टायग्रिसच्या कलाकाणुकोशिकेतील सायटोक्रोम बी जणुकाचे विश्लेषण केले गेले. शिवाय दोन बिबट आणि एक वाघाचे सायटोक्रोम बी जणूकाचे नमुणे अनुक्रमीत केले गेले. प्राप्त झालेल्या जनुक अनुक्रमाचे क्रोमास (२.१.८), क्लस्टल डब्लु, बायोइडिट, बाल्स्टएन व वृक्षवंशावळ मेगा ७ या जैवमाहिती तंत्रज्ञानाच्या साफ्टवेअर वापरून प्रथक्करण करण्यात आले. कलाकाणुकोशिकेतील सायटोक्रोम बी चे बहुमिश्रीत शृंखला अभिक्रियेद्वारे विश्लेषण केले असता १९ नमुन्यामध्ये १५६ बीपी पॅथेरा पारडूस (बिबट) आणि २७१ पॅथेरा टायग्रिस (वाघ) वर्धित झाल्याचे

आहढळले. ब्लास्टएन द्वारे कलाकाणुकोशिकेतील सायटोक्रोम बी आंशिक अनुक्रमाचे विश्लेषण पॅथेरा पारडूस आणि पॅथेरा टायग्रिस मूळ प्रजातींशी ९९ टक्के जुळणारे आहेत असे दिसून आले. वृक्ष वंशावळीमध्ये सुद्धा त्या अणुक्रमांचा समावेश त्या त्या गटांमध्ये झाल्याचे दिसून आले. सध्याच्या अभ्यासातून असा निष्कर्ष काढला विष्ठेपासून कलाकाणुकोशिकेतील सायटोक्रोम बी जनुकाचे प्रजाती विशिष्ट दर्शके वापरून पॅथेरा पारडूस आणि पॅथेरा टायग्रिस यांची यशस्वीरीत्या ओळख करता येऊ शकते. तसेच विष्ठा वापरून या प्रजातींच्या हालचाललीवर लक्ष ठेवण्यासाठी, न्यायवैदकीय पद्धतीने जप्त केलेल्या नमुन्यांची, मानव–पशु विरोधा भासांच्या खटल्यामध्ये सुद्धा वापर होऊ शकतो.