TAMIL NADU FOREST DEPARTMENT

ADVANCED INSTITUTE FOR WILDLIFE CONSERVATION

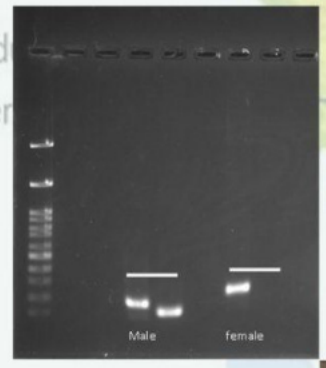
(RESEARCH, TRAINING & EDUCATION)

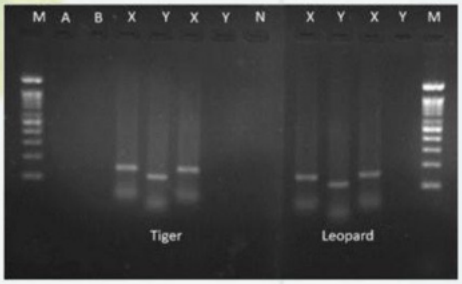
VANDALUR - 600 048

PROJECT COMPLETION REPORT

USE OF MOLECULAR TECHNIQUES FOR IDENTIFICATION OF SPECIES AND GEOGRAPHICAL ORIGIN OF ORGANISMS FROM SEIZED PRODUCTS IN WILDLIFE TRADE AND USE OF THE SAME TO CREATE IN- HOUSE DNA REFERENCE REPOSITORY/ DATABASE

Theme: Application of forensic science in wildlife crime investigations and in enhancement of species conservation





Annual Plan of Operations (APO)
(2020-21)

Tamil Nadu Forest Department

ADVANCED INSTITUTE FOR WILDLIFE CONSERVATION
(Research, Training & Education)
Vandalur, Chennai – 600 048.

Project Completion Report
On

'Use of molecular techniques for identification of speciesand geographical origin of organisms from seized products in wildlife trade and use of the same to create in-house DNA reference repository/database'

[Research theme 1: Application of forensic science in wildlife crime investigations and in enhancement of species conservation]

Annual Plan of Operations (APO) Project (2020-21)

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1(a). Molecular techniques for identification of species from seized products inwildlife trade.

Background of the study

Species authentication is an essential spotlight of ecological and biological monitoring of endangered wildlife. Molecular analysis for resolving taxa has been a significant module in predicting illegal trade and regulating the in situ and ex-situ conservation programs. Present study relevant to the identification of species from seized wildlife products with a molecular approach using species-specific primers and universal primers. Another aspect of the study is to identify the geographical origin of organisms from seized products in the wildlife trade. One of the most traded parts or demanded products in the wildlife trade is elephant ivory, because of this reason current study selected the species for the research as the Asian elephant. Lots of elephant ivory seizures are recorded from South India, but in most cases its geographic origin is not yet recognized. So, itis of utmost urgency to identify the geographic origin of elephant ivory of seized stuffs.

Introduction

Wildlife DNA Forensic is the application of regular DNA forensic methods for proper identification of wildlife parts and their products. Recent advances in molecular genetic studies have generated a new and exciting range of possible applications of genetic methods to wildlife research, conservation, and management (Mitra *et al.,* 2018). With the increasing adverse effect of natural resource depletion worldwide, conservationists and environmentalists have awakened and wants to apply strict Wildlife Act all over the continents. The main focus of that act is to stop the illegal poaching, smuggling, and hunting of endangered and threatened wildlife creatures, apart from their protection in their particular niche. To apply the law, it becomes necessary to properly identify each crime exhibit up to the species level. It is a serious worldwide concern for wildlife management to stop the illegal smuggling, hunting, and poaching of wildlife, be it for their medicinal value or ornamental body parts. Wildlife Forensic Science is nothing but the application of established and accepted forensic techniques to identify the wildlife species and help to answer the legal issues related to them. It has the same task as human forensic analysis, i.e., to relate suspect, victim, and crime scene with the minute and degraded physical evidence recovered from the scene of the crime and fix the accurate wildlife offence as well as to study the phylogenetic relationship between wild animals. Hence, Wildlife Forensics is a vital branch of Forensics, which deals with the identification of species from the biological remnants. It is a wide

range of disciplines compared to human identification and takes many guises depending on the nature of the allegation.

The researchers develop new approaches for collecting, analysing, and interpreting wild confiscated biological samples in addition to generating information relevant to managing target populations. However, the progress rate of advancements in human forensics has been more gradual than wildlife forensics because of a lack of proper attention for many years (Mitra *et al.,* 2018), but wildlife crimeinvestigation is often much more complicated as compared to other investigative Sciences. There are a number of reasons or circumstances under which animals can be killed (legally or illegally), but a lack of proper species-specific identification procedures and lots more complications in identification techniques hindered the fight against wildlife crime (Johnson et al., 2014). Thus, DNA typing of non-human DNA is a fast-developing area of research and professional practice. The application of DNA typing in Wildlife Forensic Science is one of these prime uses of DNA typing and is gaining an increasing profile.

A molecular marker or a genetic marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species. Markers derived from nuclear genes are unavailable for most wildlife, but that might change. Currently, mtDNA markers dominate the wildlife area for species identification. cytochrome b (Cyt b) gene, cytochrome c oxidase subunit I (COI) gene, 12S and 16S rRNA segment and control region (D Loop) in

animals; rbcL and matK (plastid genes)in plants are the mitochondrial markers that are used for species identification (Mitra *et al.,* 2018).

The present project attempted to identify the species from unknown seized samples using species-specific and universal molecular markers to provide supportive evidence to solve many wildlife crimes in court. On the same hand, identified species sequences were used to create an in-house DNA referencedatabase for future use.

Objectives of the study

- Genetic identification of unknown wild animal species from samples collected throughout Tamil Nadu to provide supportive data in solving wildlife crimes.
- Development of a reference genetic database of various wild animals at AIWC.

Materials and Methods

Standardization of DNA extraction protocol from seized parts/others

Case and research samples received in the DNA lab of AIWC were used for the standardization protocol.

Qiagen kit method

The procedure of DNA isolation followed in the study (Qiagen DNA isolation protocol) ishighly sensitive, which provides high-yield recovery of DNA from the minimal number of samples.Two types of Qiagen kits are used for the standardization, (a) QIAmp Blood and tissue kit. Qiagen blood and tissue DNA isolation kit (Cat. No. 51104) and Qiagen fast DNA stool mini kit (cat. no.51604) are the most effective procedures than any other protocols available that can be adopted for DNA isolation from scat, blood and tissues. This kit does not need any biological extraction and yields high graded DNA for PCR amplification in a quick time (Jen et al., 2000).

Standardisation of DNA extraction from QIAmp Blood and tissue kit

DNA extraction was done, followed by the manufacturer's instructions. DNA

extraction of different types of samples was done using QIAmp. Blood and tissue kits are mentioned below:

- Tissue
- Skin
- Bloodstain (Blood stain from tree bark) (Bloodstain should be treated with PBSbuffer before starting the Quigen kit procedure)
- Cooked meat
- Shark fin
- Antler
- Bone (Powder of the bone should be treated with EDTA buffer before starting theQuigen kit procedure)
- Elephant ivory (Powder of the ivory should be treated with EDTA buffer beforestarting the Quigen kit procedure)
- Hippopotamus canine and incisor (Powder of the canine/incisor should be treatedwith EDTA buffer before starting the Quigen kit procedure)

DNA extraction protocol from blood and tissue samples.

Reagents used: ATL buffer, PBS buffer, AL buffer, Proteinase K, AW1 buffer, AW2 buffer,absolute ethanol (96-100%) and AE buffer.

Procedure

- 20 µL of Proteinase K was added into the bottom of a new 1.5 mL microcentrifuge tube.
- 200 µL of the sample extract was added into the microcentrifuge tube that contains Proteinase K. 200 µL of whole blood or already treated tissue extract was used in this step.Buffer AL of 200 µL was added to it, and the preparation was vortexed for 15 seconds.
- The vortexed sample was incubated for 10 minutes at 56^0C in a water bath.
- Absolute ethanol (96-100%) of 200 µL was added to the sample and vortexed for 15 seconds.
- Transferred the supernatant carefully to the QIAamp spin column with a collection tube of2mL without wetting the rim of the spin column. The

cap was closed properly and centrifuged for 1 minute at 8,000 rpm.

- After centrifugation, place the spin column in a new collection tube and the collection tube with filtrate obtained in the previous step was discarded.
- 500 µL of buffer AW1 was added into the QIAamp spin column and centrifuged at 8,000 rpm for 1 minute. The collection tube with filtrate was discarded and the spin column waskept in a sterile 2 mL collection tube.
- 500 µL of buffer AW2 was added into the spin column and centrifuged at 10,000 rpm for 3 minutes.
- The spin column was centrifuged again for 1 minute and the filtrate collected in the collection tube was discarded.
- Then, the spin column was placed in a sterile labelled microcentrifuge tube (1.5 mL), and 200 µL of AE buffer was added. This was incubated at room temperature for 1 minute and centrifuged for 1 minute at 8,000 rpm.
- A portion of eluted DNA was separated, sealed and stored at -20 0C in a deep freezer. Theremaining aliquots were lyophilized and stored at -70^{o} C for further use.

Standardisation of DNA extraction from Qiagen fast DNA stool mini kit

DNA extraction of scat samples of different animals can be done using a Qiagen fast DNA stool mini kit. DNA extraction was carried out by the manufacturer's instructions. Standardisation of differentscat samples using the Qiagen fast DNA stool mini kit are:

- Dung of elephant
- Scat of tiger/leopard

Stool/scat DNA extraction protocol

Reagents used: Buffer InhibitEX, Proteinase K, Buffer AL, absolute ethanol (96-100%), bufferAW1, buffer AW2 and buffer ATE.

Procedure

- 1mL of InhibitEX buffer was added to 180-220 mg of scat sample, and the preparation was vortexed continuously for 1 minute or until it became thoroughly homogenized.
- The overhead preparation was centrifuged for 1 minute at 10,000 rpm to pellet scat particles.
- 25 µL of Proteinase K was added into a new microcentrifuge tube, and 600µL supernatantrecovered from the previous step was added.
- 600µL of AL buffer was added to the preparation and vortexed for 15 seconds to thoroughly mix the AL buffer and sample to form a homogeneous solution. Then incubatedat 70°C for 10 minutes.
- After incubation, 600 µL of absolute ethanol (96-100%) was added to the lysate and vortexed thoroughly.
- After adequate mixing, 600 µL lysate was carefully transferred to the QIAamp spin columnwith a collection tube. Then the lid was closes and centrifuged at 8,000 rpm for 1 minute. Then kept the QIAamp spin column in a new 2 mL collection tube and discarded the tube containing the flow-through. Repeated this step until all lysate was loaded.
- Added 500 µL Buffer AW1 into the spin column and centrifuged at 8,000 rpm for 1 minute. Then kept the QIAamp spin column in a new 2 mL collection tube and discarded the tubecontaining the filtrate.
- Buffer AW2 of 500 µL was added to the spin column and centrifuged it for 3 minutes at 10,000 rpm. After centrifugation, discarded the collection tube containing the filtrate.
- The spin column was placed into a new, labelled 1.5 mL microcentrifuge

tube. 200µL of ATE buffer was directly added on to the QIAamp membrane and incubated it for 60 seconds at 27°C in a water bath. This was centrifuged for 1min at 10,000 rpm to elute DNA and the sample collected containing purified DNA was stored at -70°C.

Quantitation of DNA concentration in Nano-spectrophotometer.

Basically, after DNA isolation its concentration and purity deserve primary concern. Both can be measured in the Thermo Scientific Nanodrop-spectrophotometer. Before loading the DNA, the reading of spectrophotometer was set to zero by adding 1 µL of solvent used to dissolve DNA(TB/ATE/Milli-Q water) as blank. The purity of the nucleic acid is calculated in the ratio of OD260/OD280. Ratio of ~1.8 is the indication of pure DNA. To calculate the DNA concentration of a sample, following equation is used.

DNA concentration =50µg /mL x OD260 x dilution factor.

Species identification

COI, Cytochrome *b* and 12s are the selected mitochondrial regions for molecular markers for the identification of species in wildlife trade. Some molecular primers are very specific to particular species with gives fast result without going for sequencing.

Tiger specific primer

Tiger specific molecular markers (Nittu *et al.,* 2021) were standardized for the identification of any samples from tiger origin which only shows amplification in tiger. Expected band (369bp) observed in the Agarose gel

electrophoresis documentation will give the result of the query sample. Gel documentation image of amplification in tiger specific marker is given below (Figure 1a.1):

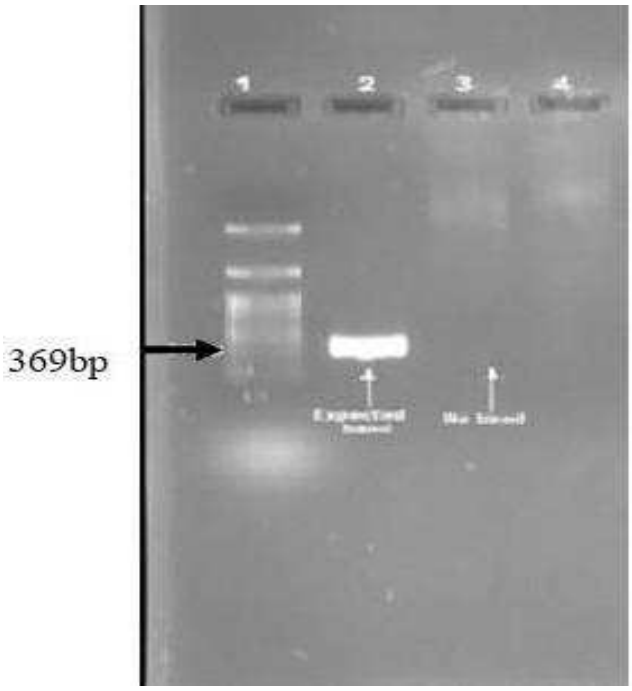

Figure 1a.1: Agarose gel showing amplification by tiger specific primer

Leopard specific primer

Leopard specific molecular markers (Nittu et al., 2021) were standardized for the identification of any samples from tiger origin which only shows amplification in leopard. Expected band (136bp) observed in the Agarose gel electrophoresis will give the result of the query sample. Gel documentation image of amplification in leopard specific marker is given below(Figure 1a.2)

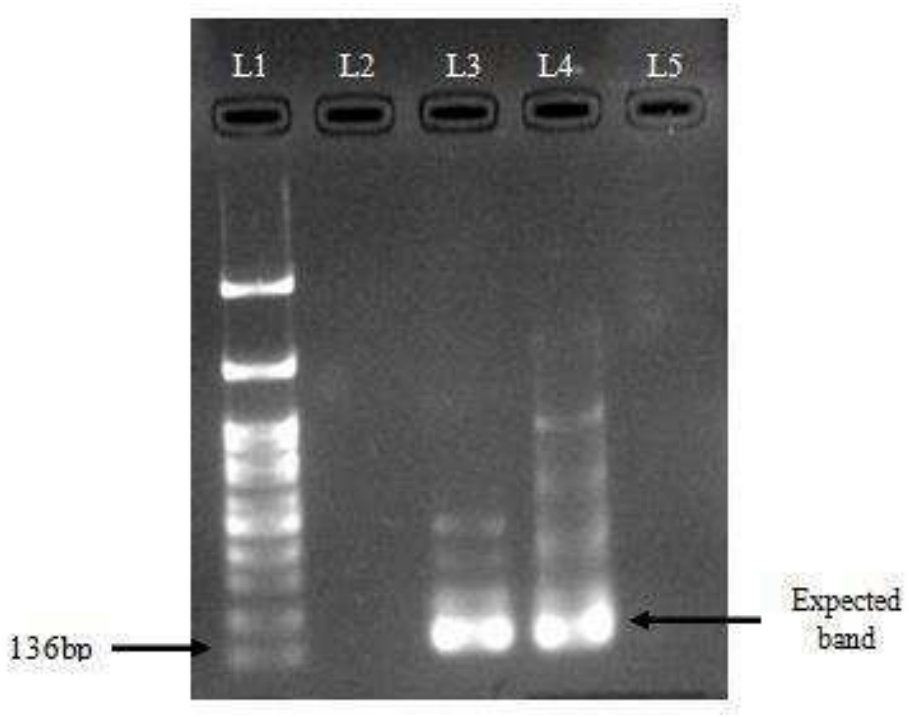

Figure 1a.2: Agarose gel showing amplification by leopard specific primerElephant specific primer

Elephant specific molecular markers (Gupta et al., 2006) were standardized for the identification of any samples from tiger origin which only shows amplification in elephant. Expected band (630bp) observed in the Agarose gel electrophoresis will give the result of the query sample. Gel documentation image of amplification in elephant specific marker is given below (Figure 1a.3):

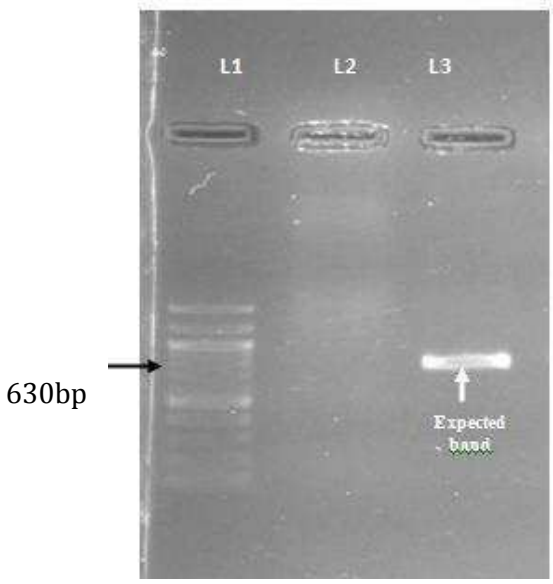

Figure1a. 3: Agarose gel showing amplification by elephant specific primer

Universal primers for species identification

COI, 12s and cytochrome *b* are the molecular markers used for the unknown species identification of case samples. After the successful PCR amplification of samples need to be sequenced for the identification of species. Nucleotide BLAST is the commonly used bioinformatic tool to check the similarity of the query sequence.

Sex identification primers

Standardization of sex identification primer for elephant

Elephant specific sex identification primers described by (Gupta *et al.,* 2006) was used for the standardization protocol. Expected band size observed in X and Y primers in the Agarose gel electrophoresis will give the gender of the query sample. Two primers which target X chromosome and Y chromosome were used in the determination of male and female elephant. After

amplification of two primers, if the Agarose gel image shows only amplification in X primers andno amplification in Y primer means it is a female elephant. On the other hand, if both X and Y primers show amplification, query sample is from male elephant. The Gel documentation image of amplification in sex identification elephant specific marker is given below (Figure 1a.4):

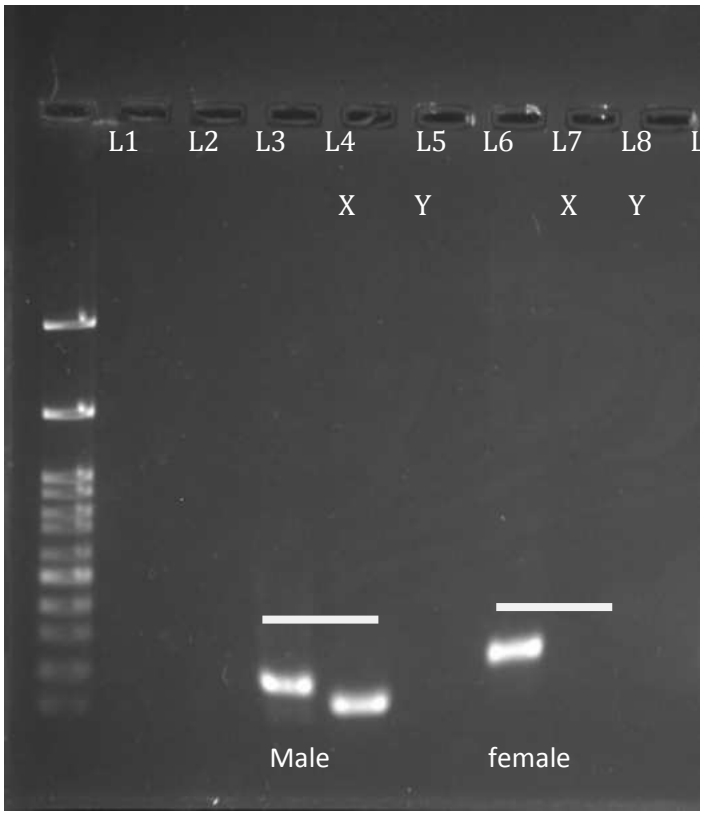

Figure 1a.4: Agarose gel showing amplification of male and female elephant.

Standardization of sex identification primer for tiger and leopard

Sex identification molecular markers (Z*fx and* Z*fy*) described by Nittu *et al* (2021) were used for the standardization. Same primer can be used for the sex determination of both tiger andleopard. Firstly, confirm the species of query

sample using Tiger/leopard specific primers and go for sex identification. Determination of male and female is based on the same method described in elephant sex identification method. The Gel documentation image of amplification of male andfemale tiger and leopard is given below (Figure 1a.5):

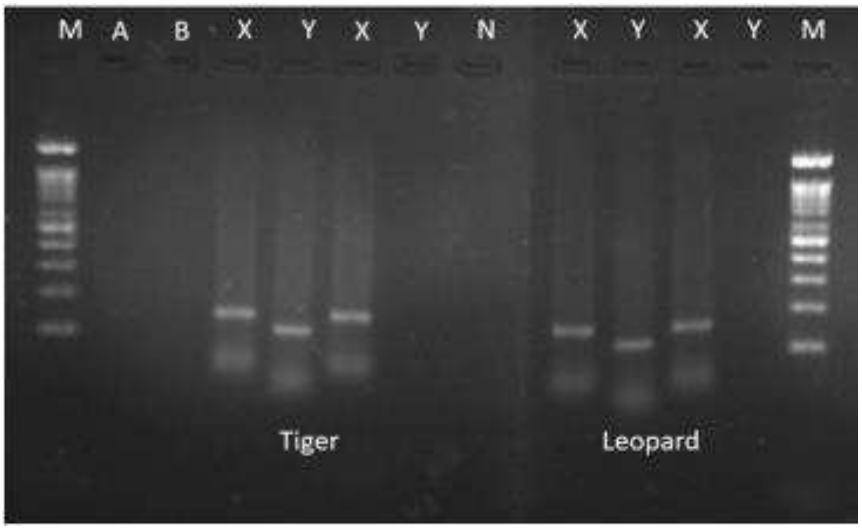

Figure 1a.5: Agarose gel showing successful sex identification. M: Marker (100–2000); A: Z*fx* Primer without template; B: Z*fy* Primer without template; N: Primer Null; X: Z*fx* amplification in both male and females; Y: Z*fy* amplification in male.

Results

Table 1a.1: Details of number of forensic case samples handled in Centre for WildlifeForensic Sciences, AIWC

Sl.No.	AIWC sample id	Sample type	Gene	Species identified
1.	AIWC/CWF/03/22/0033	Tissue	Cty *b*	Asian elephant
2.	AIWC/CWF/12/21/0025 (1)	Kidney	Cty *b*	Female Asian elephant
3.	AIWC/CWF/12/21/0025 (2)	Liver	Cty *b*	Female Asian elephant
4.	AIWC/CWF/12/21/0025 (3)	Muscle tissue	Cty *b*	Male Asian elephant
5.	AIWC/CWF/08/22/0043	Bristle hair	---	No amplification
6.	AIWC/CWF/ 03/22/0034 (A)	Head	---	No amplification
7.	AIWC/CWF/ 03/22/0034 (B)	Head	Cty *b*	Golden Jackal *(Canis aureus*)
8.	AIWC/CWF/ 03/22/0034 (C)	Head	12s	Golden Jackal *(Canis aureus*)
9.	AIWC/CWF/02/23/0012	Skin	Cty *b*, 12s	Cattle (*Bos taurus*)
10.	AIWC/CWF/02/23/0014	Blood stain	--	No amplification
11.	AIWC/CWF/03/22/0034 (D)	Fake pod	COI	Indian palm squirrel (*Funambulus palmarum)*
12.	AIWC/CWF/07/21/0015	Skin	Cty *b*	Fake tiger skin
13.	AIWC/CWF/10/22/0049	Penis	COI, Cty *b*	Sambar deer (*Rusa unicolor)*
14.	AIWC/CWF/10/22/0050	Skin	Cty *b*	Leopard (*Panthera pardus*)
15.	AIWC/CWF/01/23/0010A	Meat	Cty *b*	Spotted deer (*Axis axis*)
16.	AIWC/CWF/01/23/0010B	Meat with hair	Cty *b*	Indian gaur (*Bos gaurus*)
17.	AIWC/CWF/11/22/0054	Cooked meat	Cty *b*	Asian palm civet (*Paradoxurus hermaphroditus*)
18.	AIWC/CWF/04/23/0029 (1)	Fin	--	No amplification

19.	AIWC/CWF/04/23/0029 (2)	Fin	Cty *b*	Oceanic Whitetip shark (*Carcharhinus longimanus*)
20.	AIWC/CWF/04/23/0029 (3)	Fin	Cty *b*	Oceanic Whitetip shark (*Carcharhinus longimanus*)
21.	AIWC/CWF/04/23/0029 (4)	Fin	Cty *b*	Oceanic Whitetip shark (*Carcharhinus longimanus*)
22.	AIWC/CWF/04/23/0029 (5)	Fin	Cty *b*	Oceanic Whitetip shark (*Carcharhinus longimanus*)
23.	AIWC/CWF/09/21/0020	Tissue	Cty *b*	Tiger (*Panthera tigris*)
24.	AIWC/CWF/09/21/0018 (3)	Tissue	Cty *b*	Elephant
25.	AIWC/CWF/09/21/0018 (4)	Tissue	Cty *b*	Elephant
26.	AIWC/CWF/09/21/0018 (5)	Tissue	Cty *b*	Elephant
27.	AIWC/CWF/09/21/0018 (6)	Tissue	Cty *b*	Elephant
28.	AIWC/CWF/10/21/0024 (b)	Antler	Morphometry analysis	Spotted deer (*Axis axis*)

Table 1a.2: Details of number of research samples handled in Centre for Wildlife ForensicSciences, AIWC

Sl.No.	AIWC research id	Sample type	Gene	Species identified
1.	AIWC/RES/11-22/0128 (A)	Fake pod	---	No amplification
2.	AIWC/RES/11-22/0128 (B)	Fake pod	COI & Cyt *b*	Jungle cat *(Felis chaus)*
3.	AIWC/RES/11-22/0128 (C)	Fake pod	COI	Domestic cat (*Felis catus*)
4.	AIWC/RES/11-22/0128 (D)	Fake pod	COI & Cyt *b*	Jungle cat *(Felis chaus)*
5.	AIWC/RES/09/22/0109	Thoracic vertebrae-Bone	Cyt *b*	Male elephant
6.	AIWC/RES/05/23/0094	Muscle tissue	Cyt *b*	Elephant
7.	AIWC/RES/05/23/0093	Muscle tissue	Cyt *b*	Elephant
8.	AIWC/RES/02/23/0018	Muscle tissue	Cyt *b*	Elephant

Table 1a.3: Reference repository created from case and research samples

Sl. No	Species	Gene
1.	Asian elephant (*Elephas maximus*)	COI, Cyt *b*
2.	Golden Jackal *(Canis aureus*)	12s & Cyt *b*
3.	Domestic Cattle (*Bos taurus*)	Cyt *b*
4.	Indian palm squirrel (*Funambulus palmarum)*	COI
5.	Sambar deer (*Rusa unicolor)*	COI & Cyt *b*
6.	Spotted deer (*Axis axis*)	Cyt *b*
7.	Indian gaur (*Bos gaurus*)	Cyt *b*
8.	Asian palm civet (*Paradoxurus hermaphroditus*)	Cyt b
9.	Oceanic Whitetip shark (*Carcharhinuslongimanus*)	Cyt *b*
10.	Jungle cat *(Felis chaus)*	COI & Cyt *b*
11.	Domestic cat (*Felis catus*)	COI
12.	Crocodile	COI

Discussion

Species authentication is an essential spotlight of ecological and biological monitoring of endangered wildlife. Molecular analysis for resolving taxa has been a significant module in predicting the illegal trade and regulating the in situ and ex situ conservation programs (Sharma et al., 2016). Many approaches and assay protocols are in use to recognize the species and sex of vertebrates. The protocols to identify the species and sex, especially endangered species like tiger,leopard and elephant, gained exceptional attention as it helps to identify the specimen under quest without sequencing. Present study attempted to standardize the species-specific primers for tiger, leopard and elephant along with sex determination and identification of unknown seized wildlife articles using universal molecular markers in the DNA laboratory at AIWC to solve wildlife crimes.

All sorts of body parts of tiger, leopard and elephant can be identified using species specific primers. Results can be concluded based on agarose gel electrophoresis will be a cost effective andtime saving technique. Moreover, sex identification can also be carried out using this method by species specific gender determination primers. Use of universal primers in wildlife forensics will help to identity unknown samples under quest. Various mitochondrial markers such as cytochrome b (Cyt b) gene, cytochrome c oxidase subunit I (COI) gene, 12S, 16 and control region the commonly used for species identification. Furthermore, universal primers give a vast range of animal

species identity which includes major mammalian groups, reptiles, birds and must, therefore, prove a valuable tool for establishing species identity for forensic application.

1b. Standardisation of microsatellite primers for individual identification ofelephants:

Introduction

Microsatellite or short tandem repeat (STR) markers are suitable molecular markers for detecting genetic diversity at population and individual levels, and are, therefore, often used in molecular ecology and forensic studies (Ogden 2011). Multiplex polymerase chain reaction (PCR) amplifications of STR loci are often applied to improve the efficiency of template DNA where the quantity of a sample is limited. Among the different genetic markers used in wildlife studies are microsatellites, or simple sequence repeats (SSRs). These are regions of the genome that are madeup of short repeat sequences, consisting of one to six nucleotides (Sawaya et al., 2013), and they are widely used in studies of various organisms because of their high degree of polymorphism and their co-dominant inheritance. One of the limiting factors in the use of these markers are the development of species-specific primers (Lopes *et al.,* 2010) to resolve genetic relationships at alllevels of the population structure (Hille et al., 2002).

Microsatellites, are receiving much attention in field ecology, as the significant genetic markers chiefly in analyzing the population structure (Hettinga, *et al*., 2012), tracing the allelic traits (Luikart, *et al*., 1999), to predict the genetic variability and inbreeding status (McCarthy, *et al*., 2009), to identify the individual and to estimate the degree of relatedness between the

populations (Michalski, et al., 2011; Dobigny, *et al*., 2009) and to study the dispersal patterns of the individuals (Vidal, *et al*., 2010). The significant advantage of using microsatellite markers offer easy genotyping with less amount of DNA (Shrivastava, *et al*., 2005) which can be obtained easily from non-invasive samples like scats (Valiere, *et al*., 2000; Brinkman, *et al*., 2011). The reason behind the optimal nature of the microsatellitemarkers in studying the genetic structure of the populations is that they are highly polymorphic (Tautz and Renz, 1984), massively and randomly distributed within the genome of eukaryotes.

Present study attempted to standardize six highly polymorphic microsatellite primers of elephant for individual identification of forensic case samples received at AIWC.

Materials & methods

Known tissue samples available in the DNA lab of AIWC, used for the standardisation of microsatellite primers for elephant. Genomic DNA were extracted from the tissue samples using QIAmp Blood and tissue kit and dung samples using Qiagen fast DNA stool mini kit followed by the manufacturer's instructions. Six highly polymorphic fluorescently labelled microsatellite primers such as LAT13, LafM07, LaT05, LaT26, LaT16 and LAF12 for elephant (Archie et al., 2003) were used for the study. Microsatellite loci were amplified in 20ul PCR reactions and PCR products were electrophoresed on a 1.5 % agarose gel. Amplified PCR products were run for Fragment Length Analysis and

allelic scoring was done using the ABI Gene Scan analysis software v.3.1. Dedicated instruments and areas were used for low-copy number DNA samples in order to minimize errors in genotyping (Vidya et al., 2005). Analysis of generated peaks and allelic scoring were done using software such as Peak scanner and Geneious prime.

Result & observation

Figure 1b.1: Agarose gel showing amplification by different microsatellite primers in elephantsamples. L1 & L21- marker (100-1500bp); L2-L20 & L22-L40-elephant tissue samples.

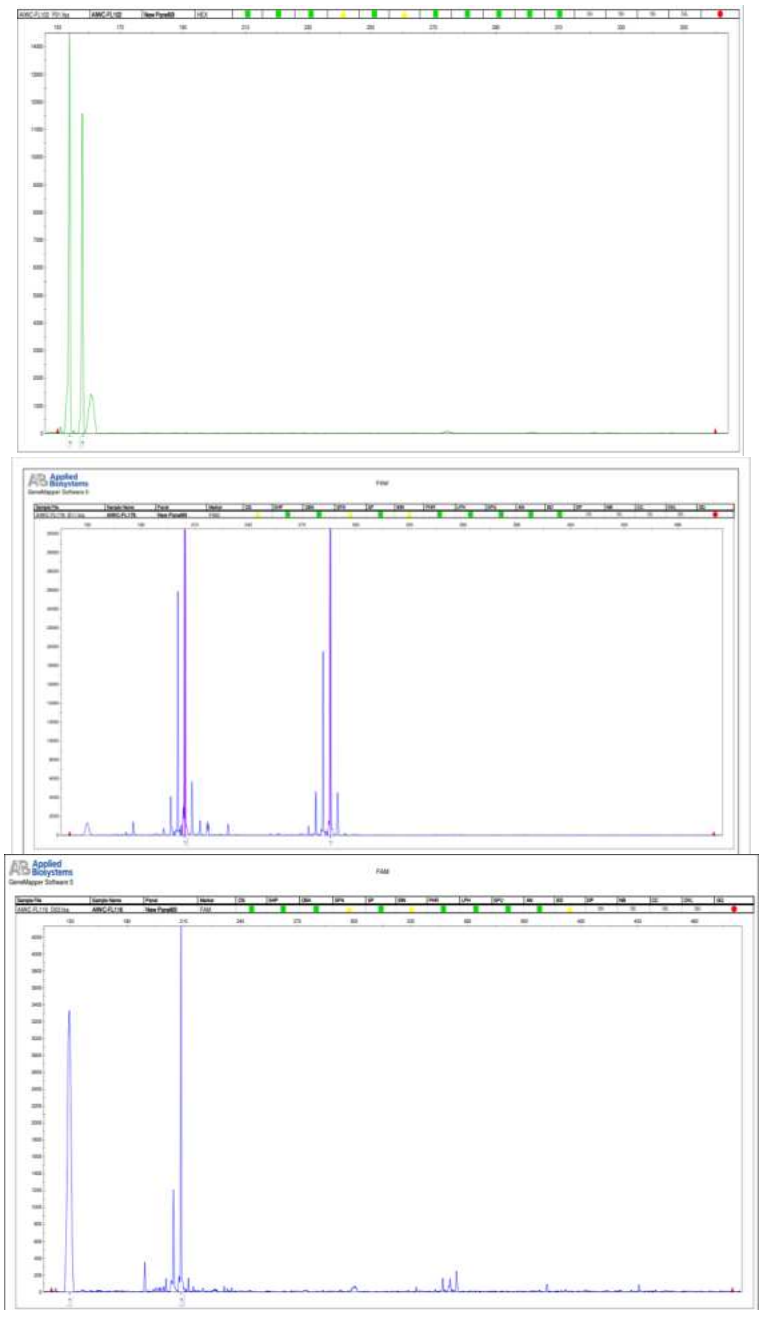

Figure 1b.2 : Peaks generated in Gene Mapper software

Table 1b.1: Table showing the allelic scoring of various tissue samples of elephant.

Sample ID	Marker	Allele 1	Allele 2
AIWC-FL288_F08.fsa	FAM	142.43	148.12
AIWC-FL303_E10.fsa	FAM	148.32	152.69
AIWC-FL313_G11.fsa	FAM	141.84	146.44
AIWC-FL307_A11.fsa	FAM	140.3	145.08
AIWC-FL304_F10.fsa	FAM	152.58	155.57
AIWC-FL286_D08.fsa	FAM	155.62	159.56
AIWC-FL290_H08.fsa	FAM	150.56	155.57
AIWC-FL292_B09.fsa	FAM	143.48	156.11
AIWC-FL314_H11.fsa	FAM	150.65	155.92
AIWC-FL306_H10.fsa	FAM	155.75	159.83
AIWC-FL316_B12.fsa	FAM	152.45	156.27
AIWC-FL291_A09.fsa	FAM	147.13	156.11
AIWC-FL296_F09.fsa	FAM	155.75	159.91
AIWC-FL301_C10.fsa	FAM	149.31	155.89
AIWC-FL311_E11.fsa	FAM	152.49	155.93
AIWC-FL309_C11.fsa	FAM	147.04	156.36
AIWC-FL285_C08.fsa	FAM	150.47	155.53
AIWC-FL294_D09.fsa	FAM	149.21	155.57
AIWC-FL317_C12.fsa	FAM	156.14	--
AIWC-FL308_B11.fsa	FAM	141.61	156.36
AIWC-FL315_A12.fsa	FAM	147.96	155.83

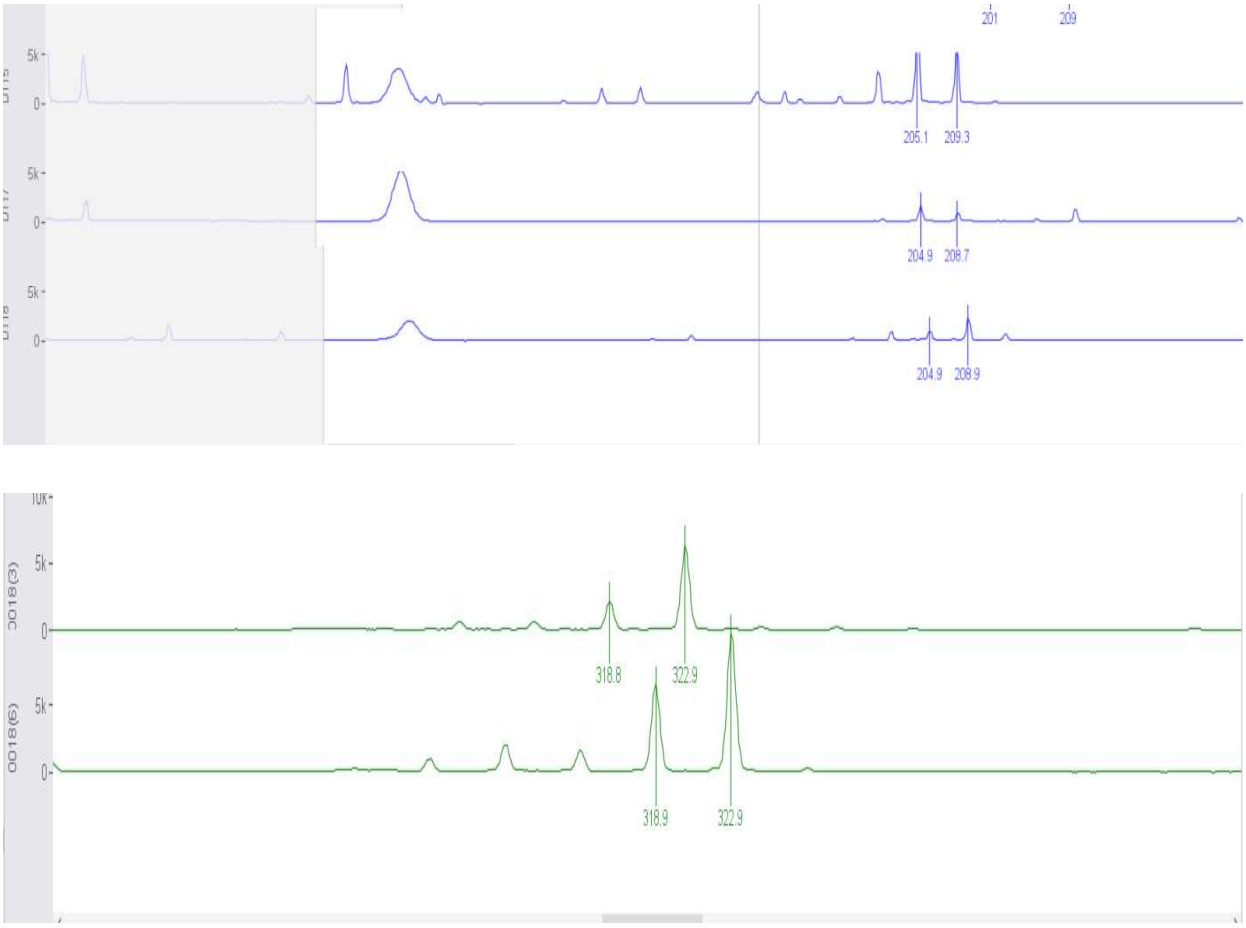

Figure 1b.3: Traces of samples generated using Geneious prime software.

Individual identification can be achieved based on the allelic scoring of each sample. Samples displaying the same or similar allelic scores indicate that they are from the same individual, (consider the score of allele 1 & 2 and compare with other samples score), as exemplified by the allelic scores of samples ID AIWC-FL-286, AIWC-FL-306, and AIWC-FL- 291, which all match (147/156). Similarly, AIWC-FL-291, AIWC-FL-309, and AIWC-FL-315 share the same allelic scores (155/159), confirming they are from the same individual

(refer to Table 1b.1). Conversely, if they exhibit different scores, it implies they belong to different individuals (refer to Figure 1b.3). By analyzing the allelic scores and the traces or peaks of all microsatellite markers, individuals can be reliably determined.

Discussion

Individual identification of elephants using microsatellite markers is a crucial technique in wildlife research and conservation. Microsatellites, also known as short tandem repeats (STRs), are highly variable regions within an organism's DNA. They consist of short, repetitive sequences of nucleotides that vary in the number of repeats between individuals. Microsatellite markers enable researchers and conservationists to identify and track individual elephants within populations. This is vital for monitoring population sizes, demographics, and movements, especially for endangered species like African and Asian elephants. Microsatellite analysis allows for the assessment of genetic diversity within elephant populations. Maintaining genetic diversity is crucial for the long-term health and adaptability of populations in the face of changing environments.

Researchers can use individual identification to study elephant behavior, social structures, and relationships within a herd. This information aids in understanding complex social dynamics and contributes to the formulation of effective conservation strategies. Another aspect of this molecular marker is conflict mitigation, in regions where human-elephant conflicts occur, knowing

which specific elephants are involved helps implement targeted strategies for mitigating conflicts, such as relocating problem individuals rather than entire herds. In captivity, individual identification is vital for managing breeding programs. It ensures that genetic diversity is maintained and that closely related individuals do not reproduce, which can lead to genetic problems.

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) regulates international trade in endangered species. Individual identification using microsatellite markers helps ensure compliance with CITES regulations by confirming the origin of seized ivory or other elephant products. Microsatellite markers are essential for scientific research on elephant populations. They provide the data needed to answer critical questions aboutgenetics, ecology, and behavior.

1c. Geographical origin identification of wild elephants ofTamil Nadu

Introduction

Identification of the geographic origin from the genetic source of an organism has an increasing significance of applications in the field of wildlife forensics and biology. This information is used for the sketching of convicts and victims and detecting the poaching location (Guillot et al., 2016). Moreover, information such as pattern of migration, gene flow of the population and different connectivity between the natural populations can also be gathered (Kremer et al., 2012). To identify the species from an unknown specimen and the identification ofthe origin of the seized sample has equal importance in case of forensics especially for the endangered species. Earlier inductively coupled plasma mass spectrometry (ICP-MS) and isotope ratio mass spectrometry (IRMS) are the two isotope techniques used in forensic science to identify the origin of a sample by incorporating it into the tissues over biological method (Tobe, 2009). These methods need the radio-isotope information of the locality which is not possible in a vast country like India. The isotope tracing (Van der Merwe et al., 1990; Ziegler et al., 2016) and X- ray fluorescence techniques (Kautenburger et al., 2004) are used in the geographic identification of elephant ivory seizures in wildlife forensic. Molecular biology offers information about a population through the microsatellites and the single nucleotide polymorphism.

At present this is a costly exercise and microsatellite evaluation is developed only to major conservation blocks alone. To determine the geographic origin of seized wildlife forensic parts or their derivatives a combination of molecular genetic tool with geo-location baseline information may be used(Mondol et al., 2015; Wasser et al., 2015). SNPs (Single nucleotide polymorphism) is the robust tool for generating DNA profiles for wildlife forensic references of endangered species. Studies by Wasser et al. (2015), were the first DNA based method for the identification of geographic location of origin of ivory in African elephants.

Mitochondrial DNA is inherited through mother and have haploid nature. Mitochondrial DNA have prokaryotic nature and its coding and non-coding regions undergo fast variation or divergence than the nuclear intron regions. The term haplotype is used to denote the single nucleotide variations in the genes of these regions. Stabled regions in the mitochondrial haplotype will provide a clue related to the origin of the elephant parts which is seized. Identification of highly variant regions can be used for detecting a particular meta-population. It forms a cost- effective and easer technique to recognize the origin of the seized wild elephant parts.

The present study attempted to establish a DNA profile for wild elephants in Tamil Nadu, solely relying on SNPs of mitochondrial DNA to determine the geographical origin of confiscatedelephant parts.

Materials & methods

Sample source:

Known tissue samples of wild elephants in AIWC and dung samples collected from Anamalai tiger reserve, Mudumalai tiger reserve, KMTR and Sathyamangalam tiger reserve wereused for the creation of DNA profile of wild elephants of Tamil Nadu.

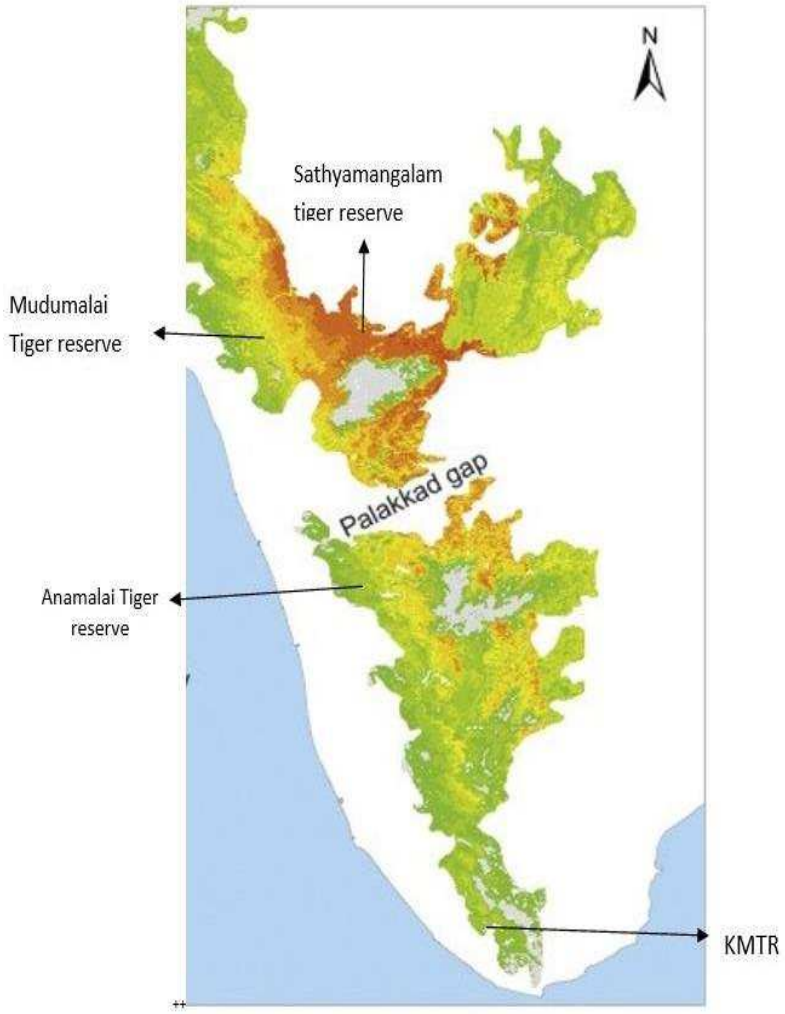

Figure 1c. 1: Map showing sampled protected areas of Tamil Nadu.

Method of sample collection and DNA extraction

DNA extraction of tissue samples were done using QIAmp Blood and tissue kit followed by the manufacturer's instructions. The dung samples from the field were collected in sterile falcontubes containing absolute ethanol, which helps to prevent the degradation of DNA by nuclease activity through fungal attack. The details such as name of the forest range, GPS location and dateof the dungs were recorded. The outer layer of the dung was collected for DNA analysis, as it may contain mucus epithelial cells of colorectal origin. DNA extracted of elephant dung samples was done using Qiagen fast DNA stool mini kit.

Total of 136 samples were used for the study which includes tissue and dung samples. PCR amplification was carried for all samples and amplified samples were sequenced. Sex determination was also carried out in all samples. Then aligned in bioinformatic software 'MEGA X', and Phylogenetic tree was created based on the sequences. Case samples from elephant origin(ivory) were also used for the study to match the location of its geographical origi

Result

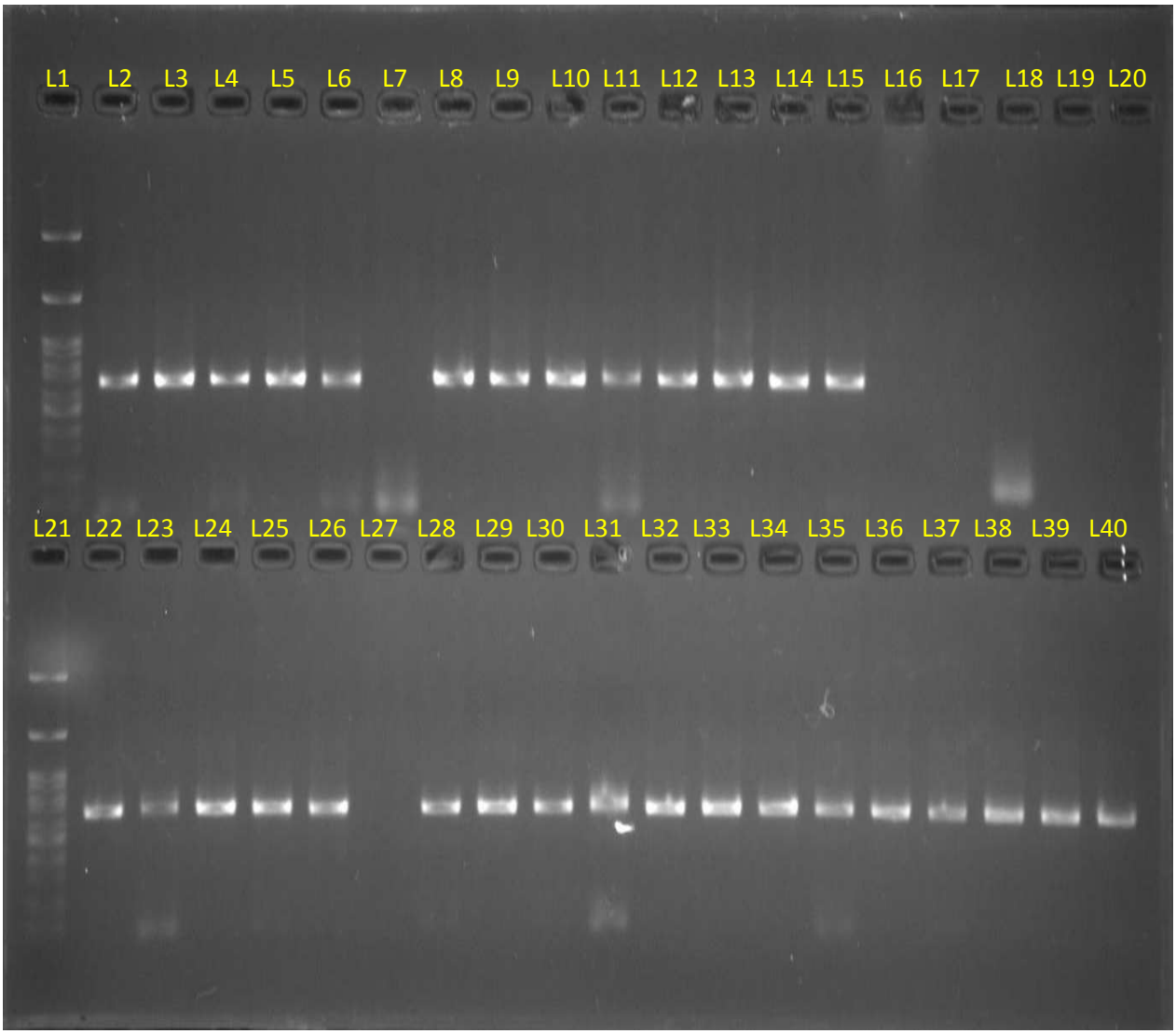

Figure 1c.2 : Agarose gel showing amplification by elephant specific primers. L1- marker (100- 1500bp); L2-L20-elephant dung sample; L21-marker; L22-37 elephant tissue sample; L38- elephant foot sole; L39-L40 elephant ivory.

Phylogenetic tree of four tiger reseres of Tamil Nadu.

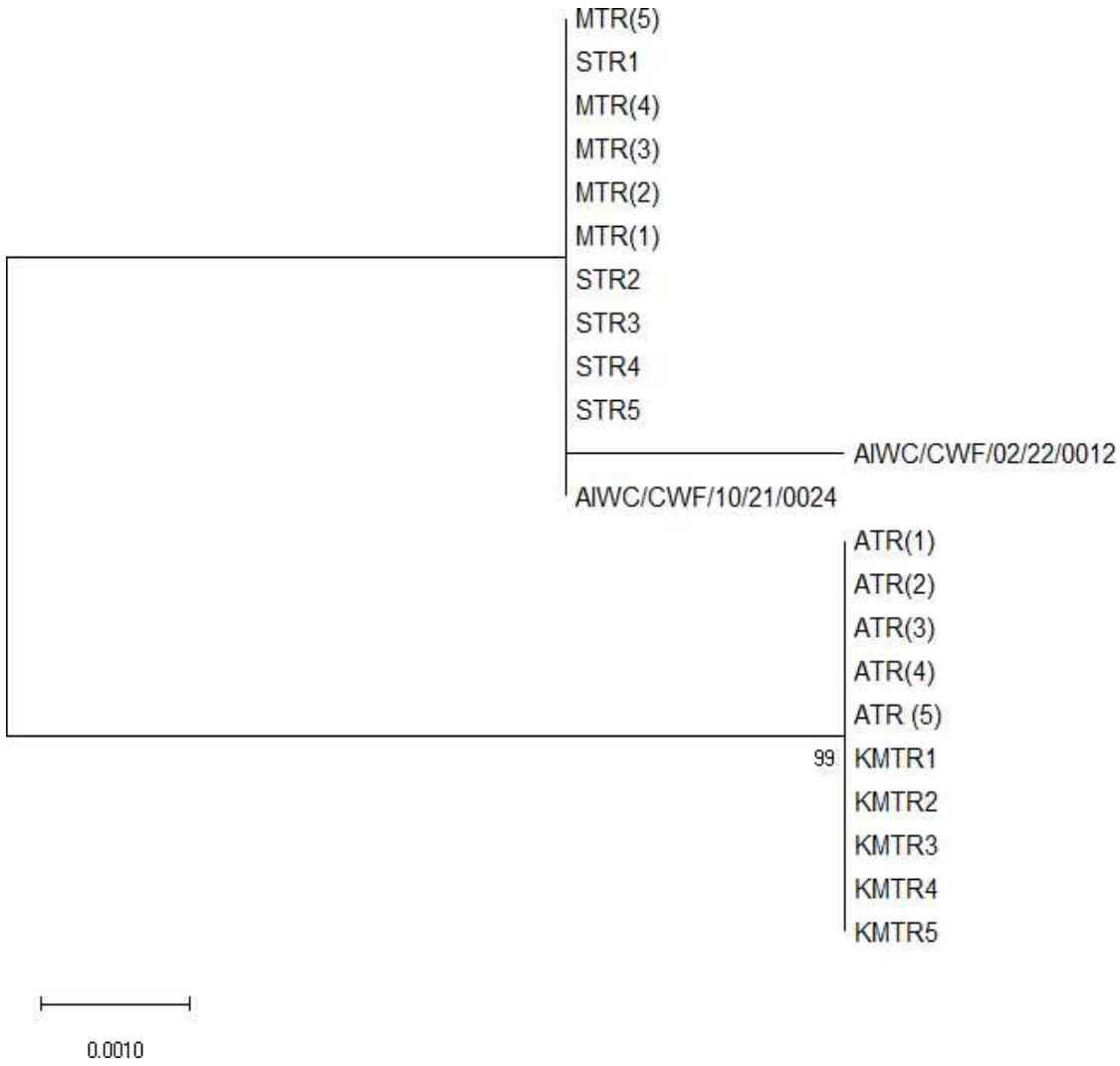

Figure 1c.3: Phylogenetic tree of four tiger reserves of Tamil Nadu (Anamalai tiger reserve (ATR); Mudumalai tiger reserve (MTR); Kalakadu-Mundanthurai tiger reserve (KMTR); Sathyamangalam tiger reserve (STR)) and forensic case samples of two ivory received at AIWC AIWC/CWF/02/22/0012 and AIWC/CWF/10/21/0024.

AIWC/CWF/10/21/0024

AIWC/CWF/02/22/0012

Figure 1c.4: Photographs of case samples (elephant ivory) received at AIWC, Morphometry lab

Observation

The present study conducted analysis of mitochondrial sequences of wild elephants of Tamil Nadu, which identified two distinct haplotypic elephant populations across four tiger reserves in Tamil Nadu. These findings revealed that there are five unique haplotypes in these populations, distinguishing Mudumalai and Sathyamangalam from Anamalai and KMTR populations. These two distinct haplotypic populations are located on the north and south sides of the Palakkad gap. Consequently, they are referred to as the 'Northern side haplotype' and the 'Southern side haplotype' of the Palakkad gap.

The 'Northern side haplotype' encompasses Mudumalai and Sathyamangalam (comprisingthe Nilgiri population), indicating connectivity not only within the Nilgiri population but also with other northern elephant populations such as Bandipur in Karnataka and Wayanad in Kerala. Conversely, the 'Southern side haplotype' (comprising Anamalai and Sathyamangalam) may exhibit connectivity with adjacent populations in Kerala. Moreover, the observed gene flow between the Mudumalai and Sathyamangalam populations and between Anamalai and KMTR populations suggests that the current contiguous corridors facilitate healthy gene flow between these populations.

However, it's important to note that this study exclusively focuses on elephant populations in Tamil Nadu, limiting its ability to differentiate Tamil Nadu's elephant populations from those in adjoining states. Therefore, the identification of the geographic origin of seized ivory or any elephant samples

can be based on the concept of the 'Northern side haplotype' and 'Southern side haplotype' of the Palakkad gap. This technique has been successfully applied to two forensic casesinvolving ivory samples received at AIWC, both of which were found to belong to the 'Northern side haplotype' of the Palakkad gap (Figure 1c.3).

Discussion

The distribution of the endangered Asian elephant has contracted over the past two to threemillennia to approximately 6% of its historical range (Vidya et al., 2004). Elephant populations inIndia are affected by habitat fragmentation and loss, human–elephant conflict, and poaching for ivory. Elephants in southern India are distributed along the Western and Eastern Ghats Mountain ranges, considered an important core area for the long-term conservation of the species (AERCC, 1998). The elephant distribution in southern India covers 39 500 km2 (AERCC, 1998; Bist, 2002), north and south of the Palghat Gap, along the Western Ghats and the Eastern Ghats mountain ranges. The northern end of the elephant distribution in southern India is restricted to pockets of approximately 260 and 50 elephants. The Nilgiris-Eastern Ghats (Nilgiris) population, which extends over part of the Western and the Eastern Ghats, is the world's single largest contiguous population of Asian elephants (AERCC, 1998), with an estimated 9400 individuals. The Anamalai population lies to the south of the Nilgiris, separated from it by the Palghat Gap. The gap is situated south of the junction of the Eastern and the Western Ghats ranges (at 101310 N

latitude and longitude 761450 E, 180 m above msl) and is the only major discontinuity in the Western Ghats chain of mountains. It is about 40 km wide and fairly steep, the Nilgiri and Anamalai blocks risingto over 2000 m above msl on either side of the gap.

Forests in the Palghat Gap were probably first cleared by humans when farming was established in southern India ca. 2000BC (Allchin and Allchin, 1997), and it is only in the past few centuries that high-density human settlements would have excluded elephants from the gap. The gap is thought to be a Precambrian shear zone with geologically recent faulting events (Rao et al, 2002). However, the role of a palaeo river system and/or marine incursion in shaping the gap havealso been hypothesized (Subramanian and Muraleedharan, 1985). The Palghat Gap is considered a phytogeographic barrier, the Nilgiri and Anamalai regions having dissimilar floristic compositions (Subramanyam and Nayar, 1974). In addition, six species of passerine birds have alternate subspecies distributions on either side of the gap (Ali and Ripley, 1987). If the Palghat gap was indeed a barrier to gene flow, it is likely to have molded the genetic structure of other independently evolving species (Avise et al, 1987), thus exhibiting a concordance across taxa.

Based on the Palakkad gap, the present study identified two metapopulations of wild elephants in Tamil Nadu. Comparing a query sample with the SNP profile of Tamil Nadu's wild elephants provides a clear understanding of the sample's origin. This technique is more reliable than

microsatellite markers due to its cost-effectiveness and accuracy. The present method relies solely on Sanger sequencing, ensuring precision, speed, and cost-effectiveness. This tool is an effective for the identification of seized sample's origin and useful for the forest department officials for the better conservation of elephant populations in the Tamil Nadu.

References

Ali, S. and Ripley, S.D. (1987). Compact Handbook of the Birds of India and Pakistan: Together with those of Bangladesh, Nepal, Bhutan and Sri Lanka, 2nd edn. Oxford University Press: New Delhi.

Allchin, B. and Allchin, R. (1997). Origins of a Civilization: The Prehistory and Early Archaeology of South Asia. Viking: New Delhi.

Archie, E.A., Moss, C.J. and Alberts, S.C. (2003). Characterization of tetranucleotide microsatellite loci in the African savannah elephant (*Loxodonta africana africana*). *Molecular Ecology Notes*, *3*(2), pp.244-246.

Asian Elephant Research and Conservation Centre (1998). The Asian Elephant in Southern India: A GIS Database for Conservation of Project Elephant Reserves. Asian Elephant Research and Conservation Centre: Bangalore.□38

Avise, J., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E. et al. (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between genetics and systematics. Annu Rev Ecol Syst 18: 489–522.

Bist, S.S. (2002). An overview of elephant conservation in India. Indian Forester 128: 121–136.□

Brinkman, T.J., Person, D.K., Chapin III, F.S., Smith, W. and Hundertmark, K.J. (2011). Estimating abundance of Sitka black-tailed deer using DNA from fecal pellets. *The Journal of Wildlife Management*, *75*(1), pp.232-242.

Guillot, G., Jónsson, H., Hinge, A., Manchih, N. and Orlando, L. (2016). Accurate continuous geographic assignment from low to high density SNP data. *Bioinformatics*, *32*(7), pp.1106-1108.□

Gupta, S.K., Thangaraj, K. and Singh, L., 2006. A simple and inexpensive molecular method for sexing and identifying the forensic samples of elephant origin. *Journal of Forensic Sciences*, *51*(4), pp.805-807.□

Hettinga, P.N., Arnason, A.N., Manseau, M., Cross, D., Whaley, K. and Wilson, P.J., 2012. Estimating size and trend of the North Interlake woodland caribou population using fecal-DNA and capture–recapture models. *The Journal of Wildlife Management*, *76*(6), pp.1153-1164.□

Hille A, Janssen IAW, Menken SBJ, Schlegel M, Thorpe RS (2002). Heterologous amplification of microsatellite markers from colubroid snakes in European natricines (Serpentes: Natricinae). J. Hered. 93(1): 63-66.

Jen, J.I.N., Wu, L.I. and Sidransky, D., 2000. An overview on the isolation and analysis of circulating tumor DNA in plasma and serum. *Annals of the New York Academy of Sciences*, *906*(1), pp.8-12.

Johnson, R.N., Wilson-Wilde, L. and Linacre, A., 2014. Current and future directions of DNA in wildlife forensic science. *Forensic Science International: Genetics*, *10*, pp.1-11.

Kautenburger, R., Wannemacher, J. and Müller, P., 2004. Multi element analysis by X-ray fluorescence: A powerful tool of ivory identification from various origins. *Journal of Radioanalytical and Nuclear Chemistry*, *260*, pp.399-404.

Lopes DM, Campos LAO, Salomão TMF, Tavares MG (2010). Comparative study on the use of specific and heterologous microsatellite primers in the stingless bees Melipona rufiventris and M. mondury (Hymenoptera, Apidae). Genet. Mol. Biol. 33(2): 390-393.

McCarthy, T.M., Waits, L.P. and Mijiddorj, B., 2009. Status of the Gobi bear in Mongolia as determined by noninvasive genetic methods. *Ursus*, *20*(1), pp.30-38.

Mitra, I., Roy, S. and Haque, I., 2018. Application of molecular markers in wildlife DNA forensic investigations. *Journal of Forensic Science and Medicine*, *4*(3), pp.156- 160.

Mondol, S., Sridhar, V., Yadav, P., Gubbi, S. and Ramakrishnan, U., 2015. Tracing the geographic origin of traded leopard body parts in the Indian subcontinent with DNA- based assignment tests. 39

Conservation Biology, *29*(2), pp.556-564.

Nittu, G., Bhavana, P.M., Shameer, T.T., Ramakrishnan, B., Archana, R., Kaushal, K.K., Khedkar, G.D., Mohan, G., Jyothi, M. and Sanil, R., 2021. Simple Nested Allele- Specific approach with penultimate mismatch for precise species and sex identification of tiger and leopard. *Molecular Biology Reports*, *48*, pp.1667-1676.

Ogden, R.O.B. (2011). Unlocking the potential of genomic technologies for wildlife forensics. *Molecular ecology resources*, *11*, pp.109-116.

Rao, T.K.G., Rajendran, C.P., Mathew, G. and John, B. (2002). Electron spin resonance dating of fault gouge from Desamangalam, Kerala: evidence for Quaternary movement in Palghat gap shear zone. Proc Indian Acad Sci (Earth and Planetary Sciences) 111: 103– 113.

Sawaya S, Bagshaw A, Buschiazzo E, Kumar P, Chowdhury S, Black MA, Gemmell N (2013). Microsatellite tandem repeats are abundant in human promoters and are associated with regulatory elements. PLOS One 8(2): e54710.

Sharma, V., Sharma, C.P., Kumar, V.P. and Goyal, S.P., 2016. Pioneer identification of fake tiger claws using morphometric and DNA-based analysis in wildlife forensics in India. Forensic science international, 266, pp.226-233.

Shrivastava, J., Qian, B.Z., Mcvean, G. and Webster, J.P., 2005. An insight into the genetic variation of Schistosoma japonicum in mainland China using DNA microsatellite markers. *Molecular ecology*, *14*(3), pp.839-849.

Tobe, S.S.S., 2009. Determining the geographic origin of animal samples. In Forensic Science in Wildlife Investigations (pp. 141-170). CRC Press.

Subramanyam K, Nayar MP (1974). Vegetation and phytogeography of the Western Ghats. In: Mani MS (ed) Ecology and Biogeography in India, Vol. 23. Dr W Junk Publishers: The Hague, pp 178–196.

Van der Merwe, N.J., Lee-Thorp, J.A., Thackeray, J.F., Hall-Martin, A., Kruger, F.J., Coetzee, H., Bell, R.H.V. and Lindeque, M., 1990. Source-area determination of elephant ivory by isotopic analysis. *Nature*, *346*(6286), pp.744-746.

Vidal-Abarca, E., Mañá, A. and Gil, L., 2010. Individual differences for self-regulating task-oriented reading activities. *Journal of Educational Psychology*, *102*(4), p.817.

Vidya, T.N.C., Fernando, P., Melnick, D.J. and Sukumar, R., 2005. Population differentiation within and among Asian elephant (Elephas maximus) populations in southern India. *Heredity*, *94*(1), pp.71-80.

Wasser, S.K., Brown, L., Mailand, C., Mondol, S., Clark, W., Laurie, C. and Weir, B.S., 2015. Genetic assignment of large seizures of elephant ivory reveals Africa's major poaching hotspots. *Science*, *349*(6243), pp.84-87.

Ziegler, S., Merker, S., Streit, B., Boner, M. and Jacob, D.E., 2016. Towards understanding isotope variability in elephant ivory to establish isotopic profiling and source-area determination. *Biological Conservation*, *197*, pp.154-163.

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