TAMIL NADU FOREST DEPARTMENT

ADVANCED INSTITUTE FOR WILDLIFE CONSERVATION

(RESEARCH, TRAINING & EDUCATION)

DEVELOPMENT OF SHORT TANDEM-REPEATS (STR)-BASED GENETIC DATABASE OF LEOPARDS FOR GEOGRAPHIC ASSIGNMENT IN WILDLIFE FORENSICS AND DETERMINATION OF POPULATION STRUCTURE

PROJECT COMPLETION REPORT

Annual Plan of Operations (APO)

2021-22

Tamil Nadu Forest Department

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On

‘DEVELOPMENT OF SHORT TANDEM-REPEATS (STR)-BASED GENETIC DATABASE OF LEOPARDS FOR GEOGRAPHIC ASSIGNMENT IN WILDLIFE FORENSICS AND DETERMINATION OF POPULATION STRUCTURE’

Annual Plan of Operations (APO)
(2021-22)

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Project Completion Report of Annual Plan of Operations 2021-22

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1.INTRODUCTION

The leopard (*Panthera pardus*) is the most widely distributed and adaptable member of the family Felidae. *Panthera pardus fusca* is absent only in the arid deserts and above the timberline in the Himalayas and is found in all forest habitats in the country (Prater, 1980; Daniel, 1996). In the Himalayas, they are sympatric with snow leopards (*Panthera uncia*) up to 5,200 m (Uphyrkina*et al.*, 2001). Leopards are very adaptive regarding habitat and dietary needs; they can be found in heavily populated and farmed areas and close to urban developments (Nowell and Jackson, 1996). Recent meta-analyses of leopard status and distribution suggest 48–67% range loss for the species in Africa and 83–87% in Asia (Jacobson *et al.*, 2016). A recent genetic study in India has reported that leopards have experienced a possibly human-induced 75-90% population decline in the last ~120-200 years (Bhatt *et al.*, 2020). This changed the species status from 'Near Threatened' to 'Vulnerable' by IUCN (Stein *et al.*, 2016).

The leopard is a solitary animal of the bush and forest. It is mainly nocturnal in habit and sometimes basks in the sun. It is an agile climber and frequently stores the remains of its kills in the branches of a tree. It feeds upon any animals it can overpower, from small rodents to waterbuck but generally preys on the smaller and medium-sized antelopes and deer. It appears to have a special liking for dogs and baboons as food. Moreover, it is protected to the highest degree in India by being included in Schedule I of the Wildlife (Protection) Act of 1972 and Appendix I of the Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES). In the Indian subcontinent poaching, habitat loss, depletion of natural prey, and conflict are major threats to leopard populations (Athreya *et al.*, 2010; Raza *et al.*, 2012). Leopards also frequently occur outside protected areas in human-dominated landscapes, increasing their vulnerability to conflict with humans (Rahalkar, 2008; Athreya *et al.*, 2010; Naha *et al.*, 2018). In areas devoid of any other large carnivore, leopards can act as an umbrella species

for biodiversity conservation. The majority of the stakeholders reported *P. pardus* as one of the major species, which is commonly poached for illegal trade (Niraj, 2009).

The population of leopard in Tamil Nadu is in the range of 828 to 908 as per the final report on the 'Status of Leopards, Co-predators and Mega herbivores in India-2018', which was released by the Ministry of Environment and Forests on 'Global Tiger Day-2021' (https://ntca.gov.in/assets/uploads/Reports/AITM/Status_Leopard_Report_2018_web.pdf).

Hunting the big cats had been a tradition for centuries in India. Leopards and tigers are still poached mainly for their skins, canines and claws. The documented leopard poaching has generally been increasing as per the statistics of WPSI (Wildlife Protection Society of India), which says that the documented deaths of leopards as well as the discovered remains of leopards like skins, bones, claws, etc. seized from wildlife traffickers has a significant count from 1994 to 1st July 2023 (https://www.poachingfacts.com/poaching-statistics/leopard-poaching-statistics/).

As for all the plants and animals, habitat loss is a cause of concern for leopards. Due to their high profile in the eyes of farmers, hunters, and poachers, it is possible that the leopard will disappear before its habitat does.

Leopard parts can be easily mistaken for tiger parts and have great value in the market. Conservationists estimate 1000 poaching deaths of big cats every year and it is crucial to consider them as highly endangered species (Anju Singh *et al.*, 2004). In advances, DNA techniques and non-invasive sampling methods can be used to monitor the populations and individuals across large landscapes including human-dominated areas. Microsatellites (short tandem repeats, STRs) are markers of choice because of their polymorphic and co-dominant nature. These strictly follow the

Mendelian inheritance and are highly reproducible (Anju Singh *et al.*, 2004). This would help in individual identification and help in developing the population's genetic structure.

Microsatellite-STR

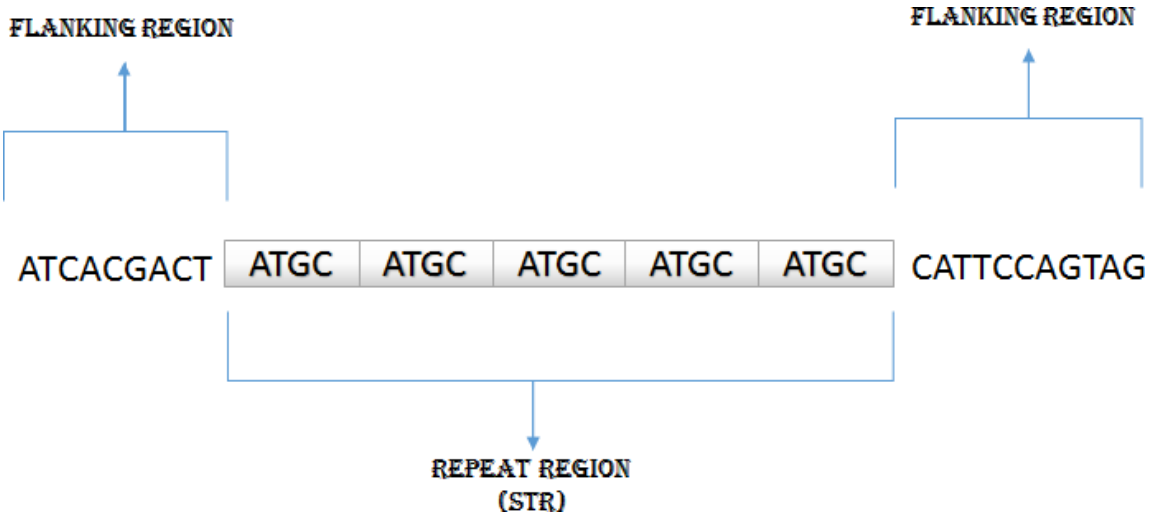


Figure 1. STR depiction

One of the DNA profiling techniques is STR-based individual identification. STR is a non-coding region that will not get into transcription and translation but is prone to variations. Unique DNA sequences display a very low mutation rate, whereas in STR, it is several orders higher in magnitude (Ellergen *et al.*, 2000). Therefore, these variations are less likely to affect an individual's phenotype. Hence, these variations are less likely to be eliminated by natural selection. STRs comprise units of bases, typically two to five bases long, that repeat multiple times. The repeat units are found at different locations throughout the genome. Every STR has multiple alleles or variants, defined by the number of repeat units present or by the length of the sequence. These repeats are surrounded by non-variable segments called flanking regions.

This STR region can be written as [ATGC]. The repeat units should be inside the bracket and the number of repeats should be outside the bracket as a subscript (Tautz *et al.*, 1993). STRs are inherited like any gene or

segment of DNA. Every individual has two alleles per STR as inherited from each parent. If the two alleles are the same, it is a Homozygous individual, and if the two alleles are different, it is a Heterozygous individual.

Microsatellites are useful genetic markers because they are highly mutant and polymorphic. The locus-specific primers are helping to amplify the microsatellites with PCR (polymerase chain reaction). Many microsatellites are located in non-coding DNA and they are biologically silent. Sometimes, microsatellite DNA can be found in both coding and noncoding regions of genes. A few genetic disorders are caused by microsatellite regions if present in the coding regions.

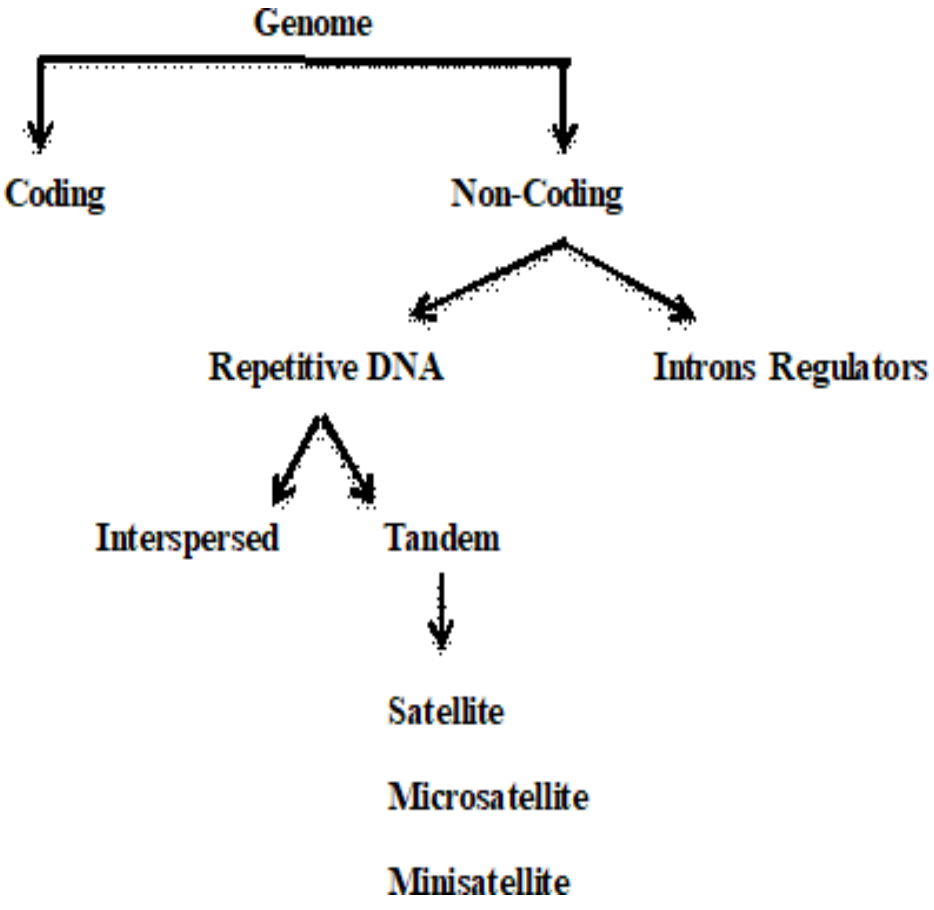


Figure2. The Genome

Repeat units AAAAAAAAAAA = (A)11 = Mononucleotide (11 bp)

GTGTGTGTGTGT=(GT)6= Dinucleotide (12 bp)

CTGCTGCTGCTG = (CTG)4 = Trinucleotide (12 bp)

ACTCACTCACTCACTC=(ACTC)4=Tetranucleotide

Homozygous microsatellite
…CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCT**A**T**CGGTACTACGTGG…(46bp)

…CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCTAT**CGGTACTACGTGG…(46bp)5"

5" Flanking region Microsatellitelocus3" Flanking region

Heterozygous microsatellite

CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCT**ATCGGTACTACGTGG…(46 bp)

CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCTCTCT**ATCGGTACTACGTGG (50 bp)

5" Flanking region Microsatellitelocus3" Flanking region

A set of short, repeated DNA sequences at a particular locus on a chromosome that vary in number in different individuals.

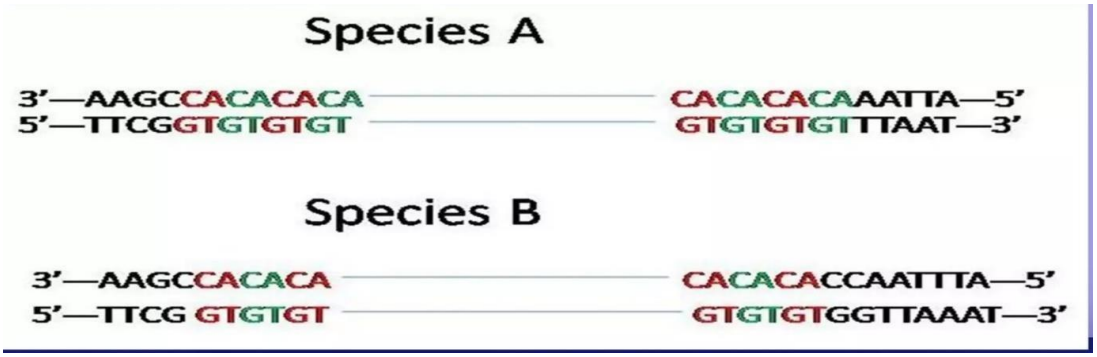


Figure 3. STR sequence

The present study, **'Development of Short-Tandem-Repeats (STR)-based genetic database of Leopards for geographic assignment in wildlife forensics and determination of population genetic structure'** helps in establishing individual identification of leopards for forensic investigation, development of STR-based genetic database for geographic assignment of leopards in Tamil Nadu, assessment of genetic diversity and population genetic structure.

2. OBJECTIVES

- Development of an in-house STR-based genetic database for geographic assignment of leopards in Tamil Nadu
- Establishing individual identification for forensic investigation
- Assessment of genetic diversity, gene flow rate, and population genetic structure of leopards in Tamil Nadu.

3. MATERIALS AND METHODS

3.1. Study area and Sample collection

As per the report of 'The National Tiger Conservation Authority (NTCA), 2018', there are 14 areas reported in Tamil Nadu which accommodate approximately 868 (828-908) leopards. Mudumalai Tiger Reserve, Sathyamangalam Tiger Reserve and Kalakad Mundanthurai Tiger Reserve support a high density of leopards. Srivilliputhur Grizzled Squirrel Wildlife Sanctuary has the highest leopard density in the Western Ghats (NTCA Report, 2018). The 14 areas are Anamalai Tiger Reserve, Coimbatore Wildlife Division, Erode Wildlife Division, Gudalur Wildlife Division, Kanyakumari Wildlife Sanctuary, Kalakad-Mundanthurai Tiger Reserve (KMTR), Kodaikanal Wildlife Sanctuary, Meghamalai Wildlife Sanctuary, Mudumalai Tiger Reserve Mukurthi National Park, Nellai Wildlife Sanctuary, Nilgiri Forest Division, Sathyamangalam Tiger Reserve and Srivilliputhur Wildlife Sanctuary.

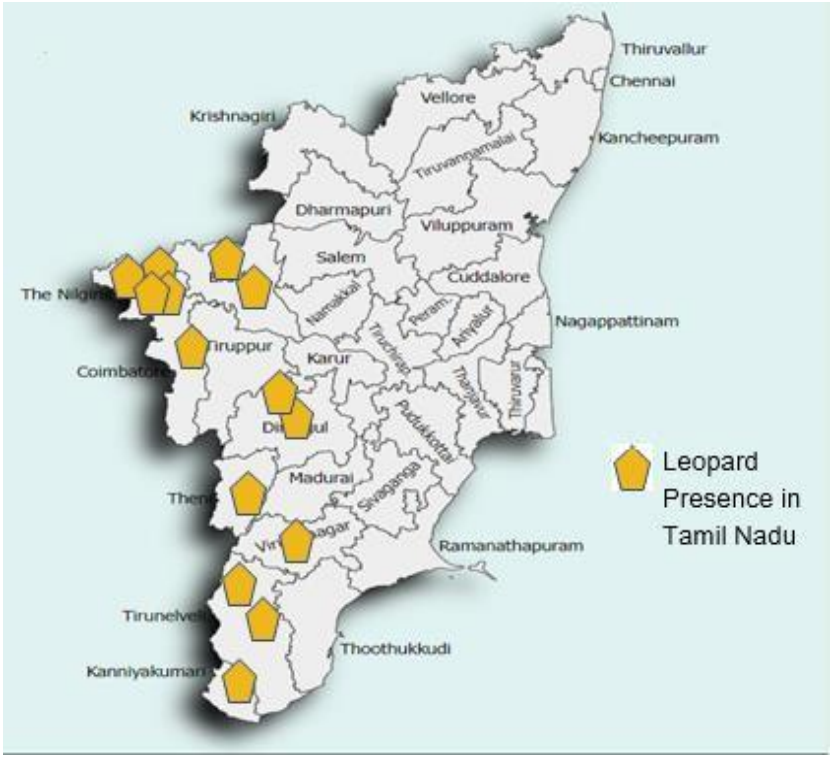


Figure 4. Map showing Leopard-presented areas in Tamil Nadu (as per NTCAreport,2018)

Since the project focused on individual identification and development of a genetic database for all the leopards in Tamil Nadu, the maximum number of leopard scat samples were collected from all over Tamil Nadu with proper permission obtained from PCCF & CWLW, Tamil Nadu (Ref No-C.No.WL5(A)/8619/2019; Dated: 18.11.2022). The field visits were carried out for scat sample collection and also for giving hands-on training to frontline staff like anti-poaching watchers (APW), forest guards (FG), and plot watchers (PW)in all tiger reserves of Tamil Nadu, regarding collection and preservation of leopard scat samples.

Field objectives

- To collect the maximum available free-ranging leopard scat samples from all over Tamil Nadu.
- To provide hands-on training on leopard scat sample collection to all the personnel like APW, FG, PW etc., who regularly monitor forest beats and ranges.
- To issue a sample collection kit for future leopard scat sample collection.
- To educate forest personnel on Leopard scat sample collection for DNA analysis.

3.2. Scat Sample Collection and Imparting Training to Forest Staff

Different beats were visited and scat samples of tiger, leopard and other animals were collected. During the field visits, the forest staff were given training on collecting and preserving scat samples. A post-monsoon census was conducted by the forest department from 15.11.2022 to 21.11.2022 in the core zone of Mudumalai Tiger Reserve. During the survey, the forest field staff collected scat samples and sent them to AIWC for research.

In Mudumalai Tiger Reserve (Buffer zone) the carnivore sign survey was conducted in alternative days (23.11.2022, 25.11.2022 & 27.11.2022). All available fresh scat samples were collected from the field and kept in ethanol vials and Zip-lock covers.

Table 1. Details of fieldwork undertaken for leopard scat sample collection and imparting training to forest field staff in different ranges of five Tiger Reserves in Tamil Nadu

Name of Tiger Reserve	Date of Fieldwork done	Places visited for the fieldwork	Total Distance covered	Activities
Sathyamangalam Tiger Reserve	6-10-2022 to 9-10-2022	Thalavadi range, Bannari beat in Bavani sagar range.	15.5 km	• Collected scat samples of Leopard • Gave training to forest staff
Mudumalai Tiger Reserve & Nilgiri division	10-10-2022 to 28-11-2022	Kargudi range, Oveli range in Gudalur division, Singara beat in Masinagudi, Sigur range in Mudumalai division, Vibudi Malai (starting point were 11.52862, 76.68467 and the endpoint was11.53932, 76.68431), Vazhaitottam (GPS coordinates were11.59016, 76.76062 at the starting point and 11.57432, 76.76982 at the end point), Anaikatti Volleyball (starting and end points were: 11.59016, 76.76062 and 11.55706, 76.75538, respectively)	39.75 km	• Collected scat samples of Leopard and Tiger • Recorded the pugmarks of leopard and tiger • Gave training to forest staff • Field activities were covered by '*Puthiya Thalaimurai*' on 12-10-2022
Anamalai Tiger Reserve	4-12-2022 to 14-12-2022	Aliyar beat in Pollachi range, Attakatti (Valparai range), Anakundhi, Varagaliyar and Yelakkaiparai, Sethumadai Pollachi and Tirupur divisions.		• Collected scat samples of Leopard and Tiger • Recorded the pugmarks of leopard and tiger • Gave training to forest staff
SrivilliputhurMegamalai Tiger Reserve (SMTR)	From 06/02/23	Devaiyar beat in Rajapalayam range, Azhagukovil beat and Watrop – II beat, at Kottaimalai Beat in Kottaimalai range, Srivilliputhur division, Vellapar Kovil beat, Gandamanur range, Megamalai division, Megamalai range, Gudalur range, Vannathiparai beat, Chinnamanur range, Thenpalai beat	30 km	• Collected scat samples of Leopard • Collected dung sample of elephant
Kalakad Mundanthurai Tiger Reserve	28-2-2023 to 9-3-2023	Papanasam range in Mundanthurai, Sivasailam beat in Kadayam range, Paatharmalai beat in mundanthurai range, Kowthalai beat, Kovilthattu beat, Upper Kodayar range, kalakad beat, Padmaneri beat in Kalakad range, Pariverisooriyan beat and Nambikovil beat in Thirukurungudi range	50 km	• Collected scat samples of Leopard, tiger, wild dog and mouse deer

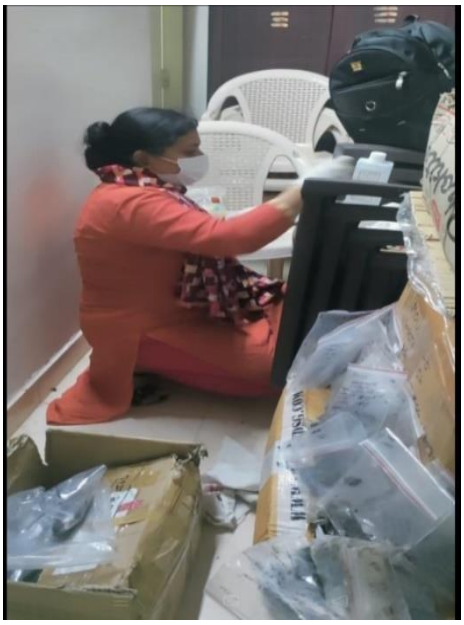


Figure 5. Transferring the field-collected scat samples from Zip-lock cover to Ethanol container

TIME FRAME - WILD ANIMAL ESTIMATION FROM 22-11-2022 To 28-11-2022

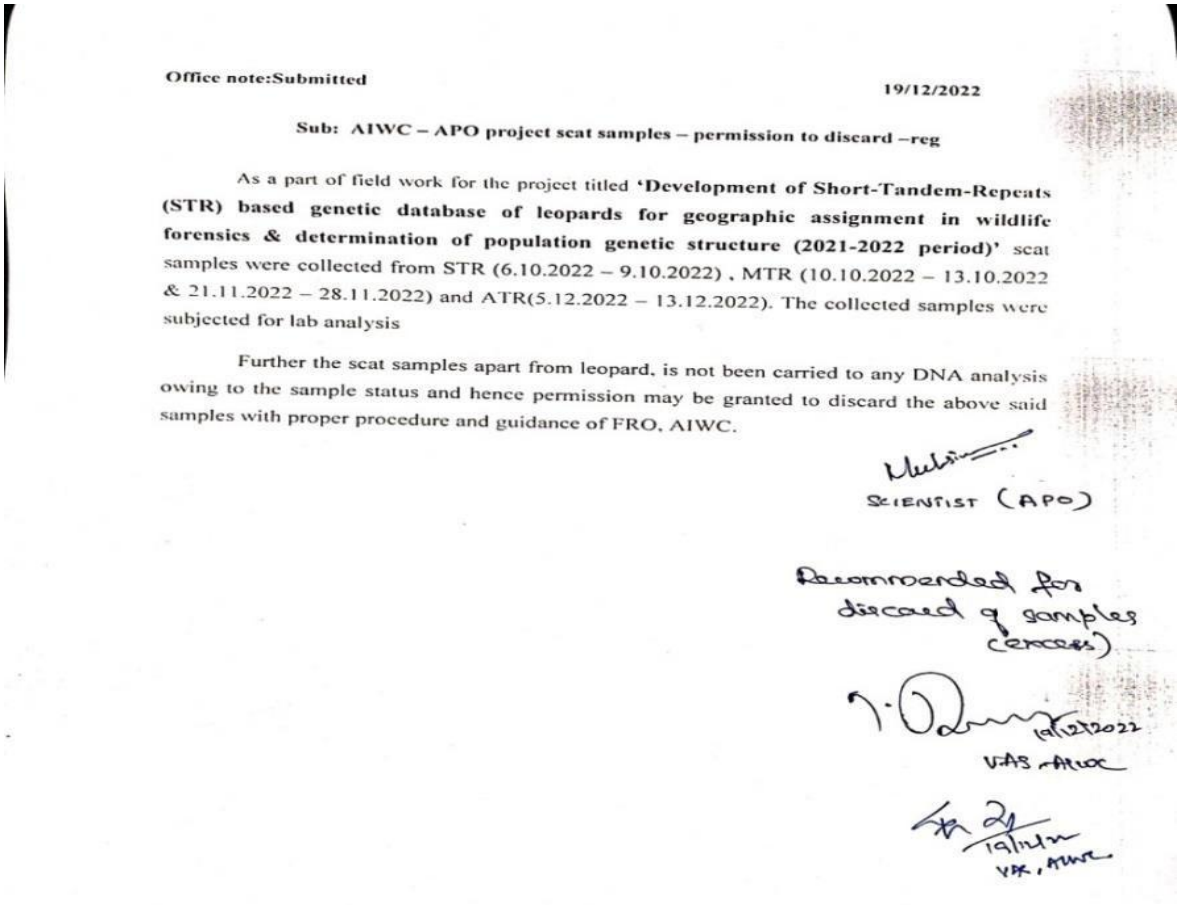
கால அட்டவணை – வனவிலங்கு கணக்கெடுப்பு - 22-11-2022 முதல் 28-11-2022

Date நாள்	Time நேரம்	Name of the Data Sheet படிவத்தின் பெயர்	Data Sheet No. படிவத்தின் அளவாளம்	Remarks குறிப்பு	Page No. பக்க எண்
22.11.2022	10:00 am	Instructions and Collection of Census Equipment's	-	-	
23.11.2022	08:00 am 01:00 pm	Carnivore Sign Survey மாமிச உண்ணிகளின் அடையாள அளவை காணல்	Data Sheet-F1 (I time) முதல் முறை	Minimum of 5.00km distance should be covered in one third of beat area	1-5
	04:30 pm 06:00 pm	Line Transect – ungulate encounter rate நேர்கோட்டில் குளம்பினங்களை கணக்கெடுப்பு	Data Sheet-F2 (I time) முதல் முறை	Species Sighted, Transect Angle, Sighting Angle and Sighting distance should be noted promptly.	6-8
24.11.2022	06:30 am 08:00 am	Line Transect – ungulate encounter rate நேர்கோட்டில் குளம்பினங்களை கணக்கெடுப்பு	Data Sheet-F2 (II time) இரண்டாம் முறை	As noted in Data sheet-F2 (I time)	9-11
	09:00 am 11:00 am	Vegetation, Human interference, Recording Ground Cover, Pellets count. தாவரங்கள் – மனித இடையூறுகள் கணக்கெடுப்பு, புழுக்கைகளின் கணக்கெடுப்பு.	Data Sheet-F3- I time 3A,3B,3C Data Sheet-F4	Tree Species, shrub and weeds name should be mentioned at the maximum & Pellets of different species should be clearly identified and counted	12-15
25.11.2022	06:30 am 08:00 am	Line Transect – ungulate encounter rate நேர்கோட்டில் குளம்பினங்களை கணக்கெடுப்பு	Data Sheet-F2 (III time) மூன்றாவது முறை	As noted in Data sheet-F2 (I time)	16-18
	09:00 am 01:00 pm	Carnivore Sign Survey மாமிச உண்ணிகளின் அடையாள அளவை காணல்	Data Sheet-F1 (II time) இரண்டாம் முறை	Minimum of 5.00km distance should be covered in one third of beat area	19-21
26.11.2022	06:30 am 08:00 am	Line Transect – ungulate encounter rate நேர்கோட்டில் குளம்பினங்களை கணக்கெடுப்பு	Data Sheet-F2 (IV time) நான்காவது முறை	As noted in Data sheet-F2 (I time)	22-24
	04:30 pm 06:00 pm	Line Transect – ungulate encounter rate நேர்கோட்டில் குளம்பினங்களை கணக்கெடுப்பு	Data Sheet-F2 (V time) ஐந்தாம் முறை	As noted in Data sheet-F2 (I time)	25-27
27.11.2022	06:30 am 08:00 am	Line Transect – ungulate encounter rate நேர்கோட்டில் குளம்பினங்களை கணக்கெடுப்பு	Data Sheet-F2 (VI time) ஆறாம் முறை	As noted in Data sheet-F2 (I time)	28-30
	09:00 am 01:00 pm	Carnivore Sign Survey மாமிச உண்ணிகளின் அடையாள அளவை காணல்	Data Sheet-F1 (III time) மூன்றாம் முறை	Minimum of 5.00km distance should be covered in one third of beat area	31-33
28.11.2022	11.00 am	Collection of data sheets and conclusion	-	Interaction and feedback	

Figure 6. Datasheet showing the details of animal survey in MTR

3.3. Disposal of inoperative scat samples

The epithelial layer of fresh scat samples was collected, and unwanted, dry and inoperative scat samples were discarded following proper procedure and guidance from FRP, AIWC. The reference letter is given below:



Office note:Submitted

19/12/2022

Sub: AIWC – APO project scat samples – permission to discard –reg

As a part of field work for the project titled **'Development of Short-Tandem-Repeats (STR) based genetic database of leopards for geographic assignment in wildlife forensics & determination of population genetic structure (2021-2022 period)'** scat samples were collected from STR (6.10.2022 – 9.10.2022) , MTR (10.10.2022 – 13.10.2022 & 21.11.2022 – 28.11.2022) and ATR(5.12.2022 – 13.12.2022). The collected samples were subjected for lab analysis

Further the scat samples apart from leopard, is not been carried to any DNA analysis owing to the sample status and hence permission may be granted to discard the above said samples with proper procedure and guidance of FRO, AIWC.

SCIENTIST (APO)

Recommended for discard of samples (excess)

19/12/2022

VAS, AIWC

19/12/22

VAS, AIWC

Figure 7. Official note for scat disposal

The training on leopard scat sample collection was successfully conducted for the forest personnel during the fieldwork, which would help the project team to receive more samples directly from the forest personnel.

3.4. Scat Sample Collection and Storage Methods

All equipment used for scat sample collection should be sterilized to prevent contamination of samples.

3.4.1. Dry Scat Collection

3.4.1.1. **Ethanol collection**: A sterile blade was used to scrape the shiny upper epithelial layer of the scat gently. This technique preserves the scat's outer characteristics.

3.4.1.2. **Silica desiccant**: In an effort to mitigate direct interaction between the silica desiccants and the epithelial layer of the scat, the desiccants were cautiously enveloped using tissue paper, creating a protective bundle. Then, utilizing a Ziplock cover, the dry scat was methodically collected. The prepared silica desiccant bundle was introduced into the Ziploc cover alongside the scat to enhance preservation further.

3.4.2. Fresh Scat Collection

When encountering fresh scat, the swab method is the best to collect the shiny upper epithelial layer. This rapid procedure captures surface features without compromising overall composition.

3.4.3. Sample Storage

The collected epithelial layer was transferred into a sterile 2ml tube containing absolute ethanol. The tube was sealed with Parafilm to prevent ethanol evaporation and contamination. For long-term storage and preservation, the samples may be kept in -20 freezer.

3.5. Documentation

Essential information, including GPS coordinates, collection dates, and environmental conditions, were recorded and documented. This contextual data provides valuable insights for future analysis.

3.6. DNA extraction

The DNA extraction was done using QIAamp® Fast DNA Stool Mini Kit. The protocol is given below:

- The scat sample was taken in a 2ml tube (it should not be more

than 1/4th of the tube).

- 1ml of Inhibit EX buffer was added to it.
- It was mixed well by vortexing and incubated at 70°C for10 min.
- Thetubewascentrifugedfor1minat 1000rpmtopelletthestool particles.
- A new 2ml micro centrifuge tube was taken and 25µlProteinaseK was added into it.
- 600µl of supernatant was pipetted from the centrifuged tube and added into the new tube containing Proteinase K.
- After that, 600µl of Buffer AL was added in to it (Proteinase K should not be added directly to Buffer AL).
- The mixture was incubated at 70°C for 20min and then 600µl of ethanol (96-100%) was added to the lysate. It was mixed well by vortexing.
- Short spin was given and 600µl lysate was carefully pipetted from the above tube and carefully applied in QIAamp spin column. The spin column was closed and centrifuged for 1min at 14000rpm.
- The filtrate was discarded and 500µl Buffer AW1 was added into the column. The column was centrifuged for 1min at 14000 rpm and the filtrate was discarded. This step was repeated one more time.
- The QIAamp spin column was opened and 500µl Buffer AW2 was added. The column was centrifuged for 3min at 14000rpm, and the filtrate was discarded. This step was repeated once.
- The spin column was opened and 500µl ethanol (96-100%) was added to it. The column was centrifuged for1min at 14000rpmandthe filtrate was discarded.
- The spin column was placed in a new collection tube and centrifuged for3min.
- The spin column was transferred into a new, labelled 1.5ml microcentrifuge tube and 60µl nuclease-free water was added directly onto the QIAamp membrane. It wasincubatedfor5min at room temperature.
- The tube was centrifuged for1min at14000rpm toelutethe DNA.

- The DNA was quantified using Nanodrop spectrophotometer.

3.7. Species confirmation

The extracted DNA was processed for species confirmation through PCR using three species-specific primers. This method was used to segregate and confirm the leopard samples from all the collected scat samples without sequencing.

The list of primers used for amplification of the target regions of the DNA are given in Table 2.1.

Table 2.1. The species-specific primers used to amplify the target regions in the scat DNA

Name of Primers and Reference	Details of Primer Sequence	Amplicon Size	Annealing Temperature (Ta)
NADH2 (Mondol *et al.* 2014)	TGTAGGYTGAATARCAGC GGGGACATTATTAGAACC	190bp	52°C
NADH4 (Mondol *et al.* 2014)	TRATAGCTGGYTGATGAC GTTTGTGCCTATAAGGAC	130 bp	45°C
LSP (Maroju *et al.*,2017)	TCCCCGCTCCATCCAACATCTCAAC CCATGTCTCTGAGAAA	277 bp	47°C
TIF (Bhagavatula & Singh,2006)	ATAAAAAATCAGGAATGGTG TGGCGGGGATGTAGTTATCA	190 bp	55°C

Table 2.2. The PCR cyclic conditions used for NADH2 & NADH4 primers

PCR stage	Temperature	Duration	No. of cycles
Initial denaturation	95°C	15 min	1
Denaturation	94°C	30 sec	40
Annealing	Ta	30 sec	
Elongation	72°C	30 sec	
Final elongation	72°C	10 min	1
Hold	4°C	∞	-

Table 2.3. The PCR cyclic conditions used for LSP and TIF primers

PCR stage	Temperature	Duration	No. of cycles
Initial denaturation	95°C	5 min	1
Denaturation	94°C	30 sec	40
Annealing	Ta	30 sec	
Elongation	72°C	30 sec	
Final elongation	72°C	10 min	1
Hold	4°C	∞	-

3.8. STR development

Specific microsatellite primers were selected from the literature (Mondol *et al.,* 2015) and were used in PCR reactions. The list of microsatellite primers is given below:

Table 2.4: Detailof STR primers

Name of Primers	Details of Primer Sequence	Amplicon Size	Annealing Temperature (Ta)
msHDZ170	ACATTGGGTGTAGAGACTACTT ATGACTTTGCTAGTTTATAGCC	84 – 94bp	55°C
msFCA441	TATCGGTAGGTAGGTAGATATA TATGGCACAAGCCTTGAAGCAA	137 153bp	–50°C
msFCA391	GAAGTTACATCAAAAATGCCTG TTCCTTCAAATTTCCATTCGAA	190 218bp	–56°C
msFCA506	GCCACCAGGTGTCAGTGTAAGC TCCGCGTGTAAGTTTAGGCGAG	192 226bp	–54°C
FCA230	AAGAATGGACTTGGGAAATGG AAACCACAACAGGCAAAAGG	98 – 106bp	52°C
FCA309	AGAGATGGGCTCAGTTGCAT CTGGTTACCCCGAATTCTCA	92 – 106bp	55°C
FCA232	ATGACCATCTCAAACTTCATGG AGCTGAGTTTGCGTTTATCATG	113 123bp	–55°C
FCA090	ATCAAAAGTCTTGAAGAGCATGG TGTTAGCTCATGTTCATGTGTCC	104 116bp	–55°C
FCA052	TGTATCCTCTGCTCCTGAAACA ACCTGTCCCAGTGCTTGTG	107 121bp	–55°C
FCA672	AAGTTGCTTGCACACACTGC TCCAAGAGCCTTTTCAGTTAGG	82 – 100bp	54°C
FCA279	AGCCAAGTAATATTCCTCTGTG GTCCATCCGCAGATGAATG	81 – 105bp	57°C
FCA126F	GCCCCTGATACCCTGAATG CTATCCTTGCTGGCTGAAGG	134 154bp	–57°C
FCA453	GCCCCTGATACCCTGAATG CTATCCTTGCTGGCTGAAGG	181 201bp	–59°C

Table 2.5. PCR condition for STR primers

Cycle stage	Temperature	Duration	No. of cycles
Initial denaturation	95°C	5min	1
Denaturation	95°C	30sec	40
Annealing	Ta	30sec	
Elongation	72°C	30sec	
Final elongation	72°C	10min	1
Hold	4°C	∞	-

3.9. Sex Identification

Table 2.6. Primers used for sex identification

ZNF	AAGTTTACACAACCACCTGG CACAGAATTTACACTTGTGCA	62°C	163-166 (Male)166 (Female)
AML	CGAGGTAATTTTTCTGTTTACT GAAACTGAGTCAGAGAGGC	59°C	194-214 (Male) 214-Female

3.10. Preparation of PCR Samples for Fragment Analysis on the 3500

- Amplified the target DNA fragment using primer pairs of which the forward was labelled with a capillary-based dye.
- The PCR product was diluted in triple distilled water.
- Prepared internal standard by adding 0.5 µl of LIZ standard to 9 µl of HiDi formamide and mixed by pipetting.
- Pipetted out 0.5 µl of the dilute PCR product into individual wells of the microtitre plate
- Transferred 9 µl of the standard/formamide mix into individual microtitre plate wells and mixed by pipetting.

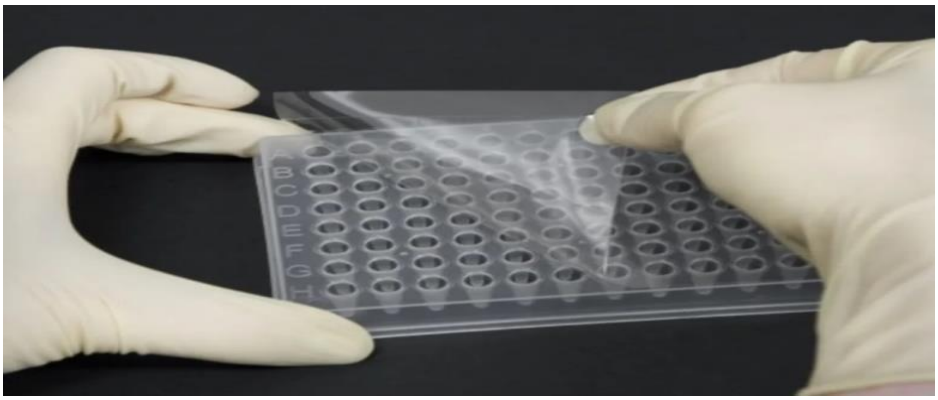


Figure 8. Plate Preparation for GenScan

3.11. Capillary electrophoresis

PCR amplification of STR regions will be done using different fluorescent-tagged primers for detection and differentiation. Capillary electrophoresis will separate the DNA fragments by size. It overpowers normal electrophoresis by working fast and being automated.

3.11.1. Working mechanism for Capillary electrophoresis

The PCR amplified products will be run through ultrathin capillary tubes filled with a polymer.

An electrical field will be applied to cause the negatively charged DNA fragments to move towards a positively charged electrode.

Larger DNA fragments move more slowly than smaller fragments.

As samples draw through the capillary tube, a laser will excite the tags and to emit the fluorescence.

The emitted fluorescence will be measured by an electronic camera.

Fragments of same size will pass the detector at the same time.

DNA ladder (running it during the experiment) will allow us to determine the sizes of alleles in DNA samples.

Computer software uses data from the detector to determine the sizes of alleles.

Figure 9. Workflow of Capillary Electrophoresis

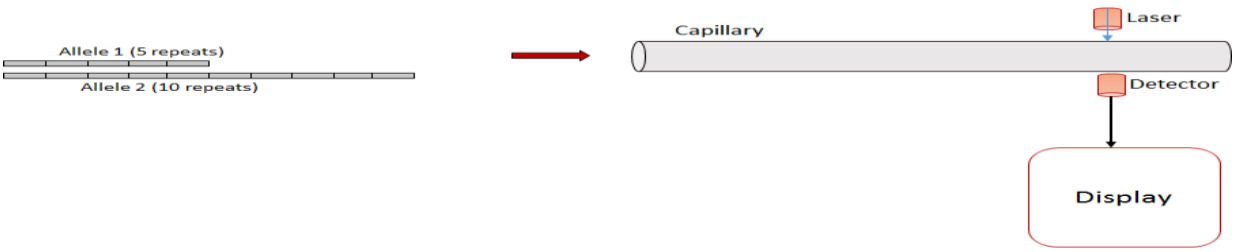
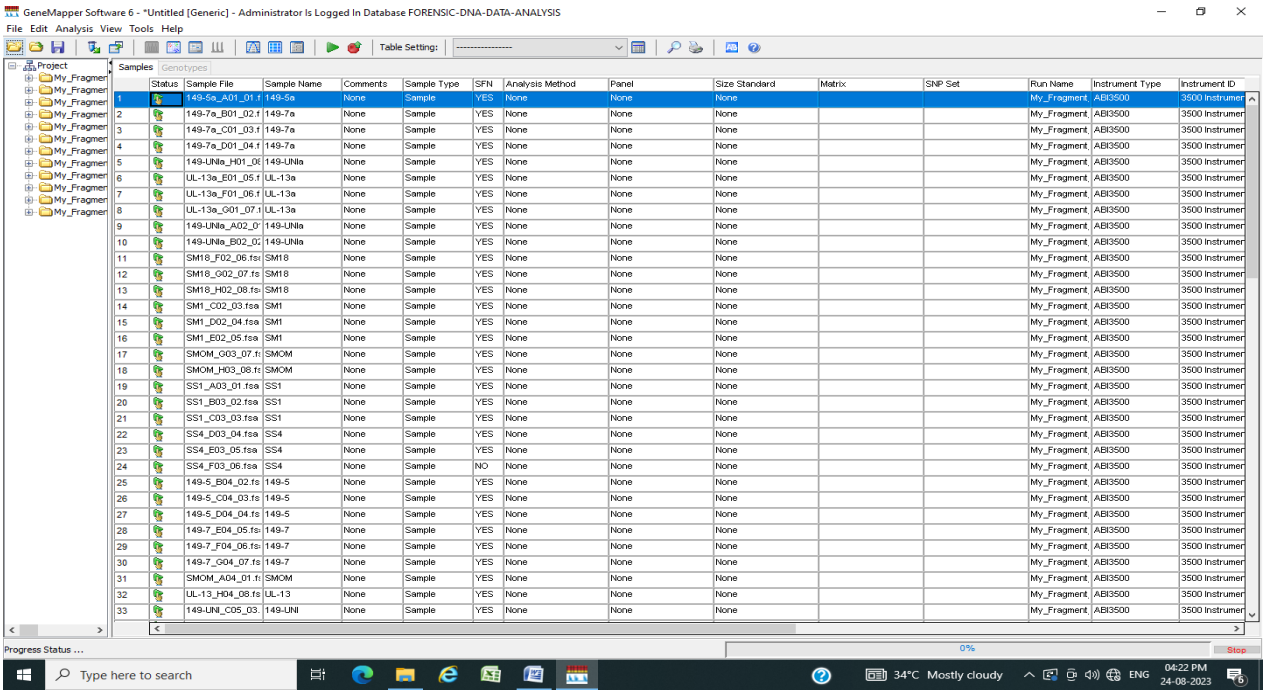


Figure 10. Depiction of Capillary electrophoresis

3.12. Data Analysis

As per the number of repeats, the fragment size varied, and the greater the number of fragments at a particular size, the higher the peak at that particular length. If only one or two repeats alone vary, the peaks will be very near because the fragment size varies with few base pairs. Even at one particular size, the amplicons can have one or two base pairs difference, so the peaks with one or two base pairs difference can be considered as a single amplicon, but the highest peak is the main one, which shows the actual size of the amplicon.



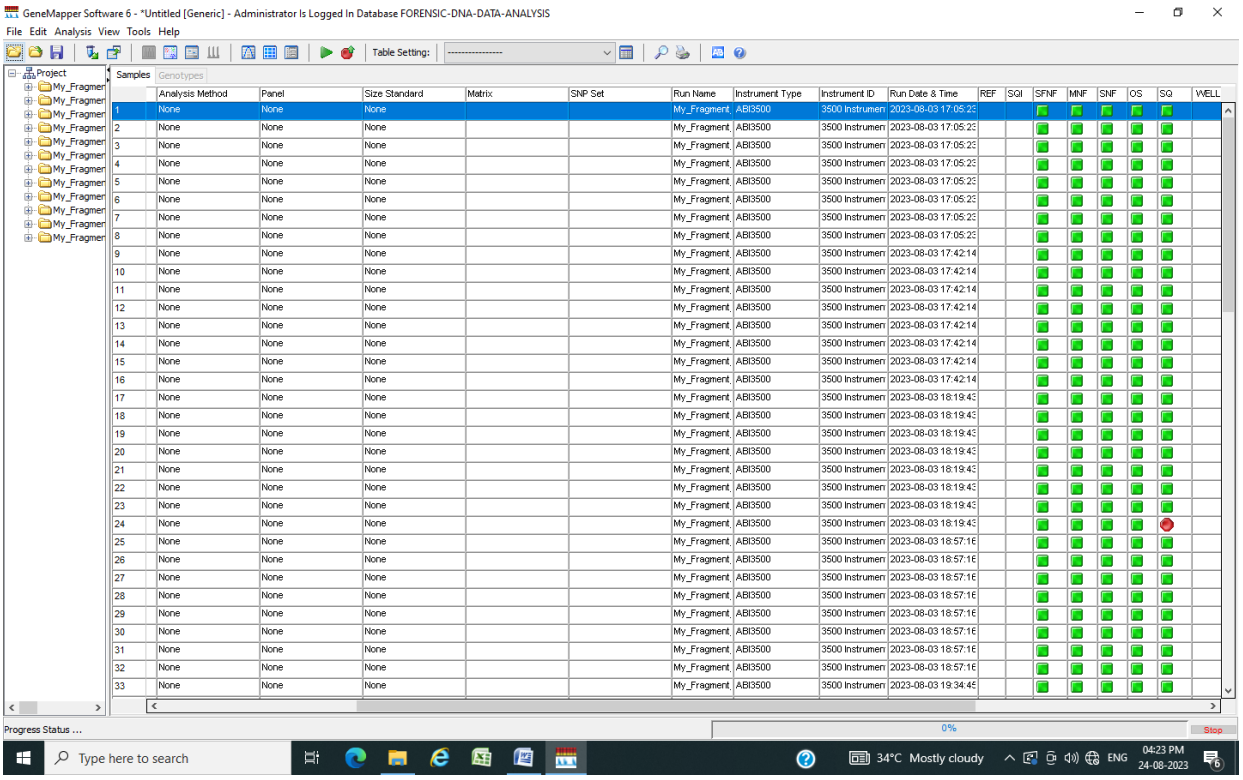


Figure 11. Data analysis with Software Gene Mapper

2.16. Raw data generation

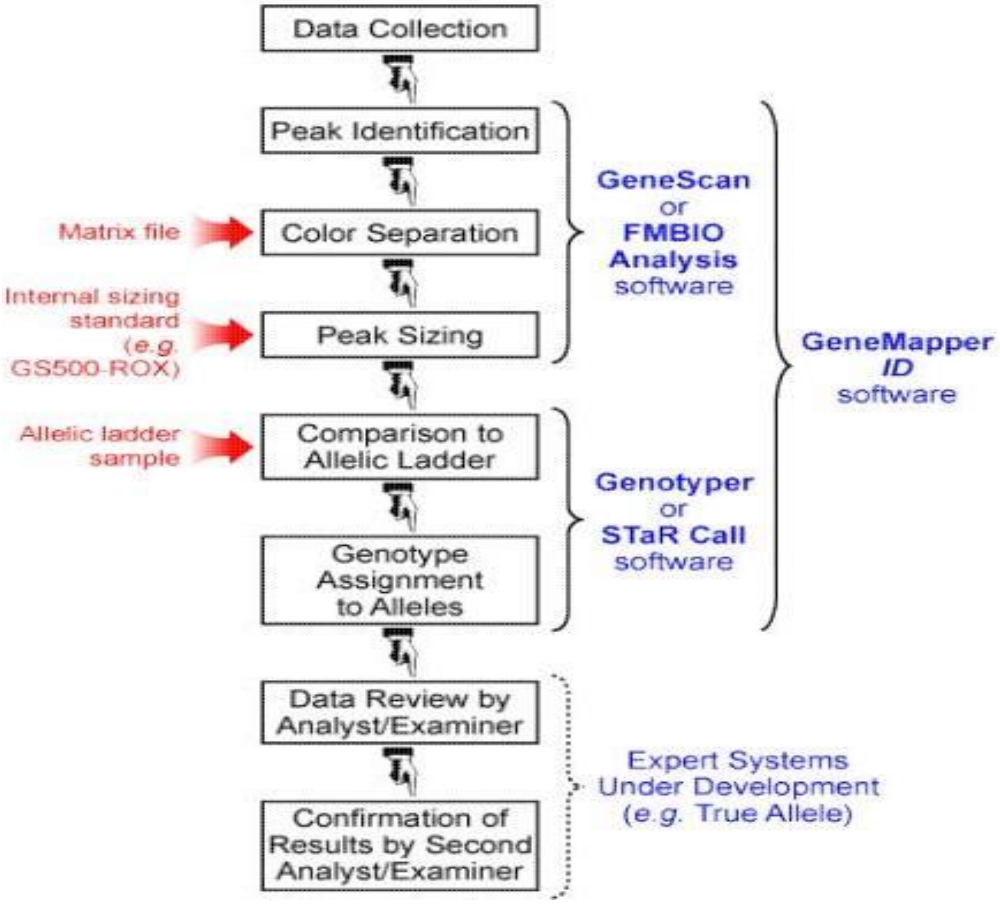


Figure 12. Data generation (Adopted From: https://www.sjsu.edu/people/steven.lee/courses/c2/s2/STR%20Data%20Analysis%20and%20Interpretation%20for%20Forensic%20Analysts.pdf)

4. RESULTS

4.1 Details of collected Scat samples

A total of 125 scat samples were collected from various Tiger reserves of Tamil Nadu bydirect field visit. Also received 36 scat samples from various Tiger reserves of Tamil Nadu during census programme (Table 4.1).

Besides leopard scat samples, the team collected scats of other animals, such as wild dogs, bears, gaur and tigers and two dung samples of elephants.

Table 4.1. Detailed count of scat samples

Tiger Reserve	Total Collected Scat (Field-collected + Received from field staff during Census)
MTR	28 +4 (32)
STR	9 +1 (10)
KMTR	20 +4 (24)
SMTR	24 +4 (28)
ATR	41 +19 (60)
Coimbatore division	0 +2 (2)
Unknown	3 +2 (5)
Total	125 +36 **(161)**

4.2 Species confirmation

Of the 161 scat samples, 38 were confirmed as leopard scats and 31 as tiger scats (Table 4.2).

Table 4.2. Total number of Leopard and Tiger scats collected from different tiger reserves of Tamil Nadu

Tiger Reserve & Division	Animal species	
	Leopard scats	**Tiger scat**
MTR	5 (2)	15 (1)
STR	0	3
KMTR	5 (2)	(2)
SMTR	5	0
ATR	21 (3)	7 (6)
Coimbatore division	0	(1)
Unknown	2	3 (2)
Total	**38 (7)**	**31 (12)**

Values in parentheses are the number of scat samples collected by field staff during the annual census programme in the tiger reserves

4.3 Species Confirmation

Totally 161 scat samples were collected from various parts of Tamil Nadu. Among them, 38 scat samples were confirmed as leopard scats, followed by PCR analysis using species-specific primers, namely NADH2, NADH4 and LSP. These primers were first standardized with known scat samples obtained from Arignar Anna Zoological Park (AAZP) and tissue samples available at the AIWC repository.

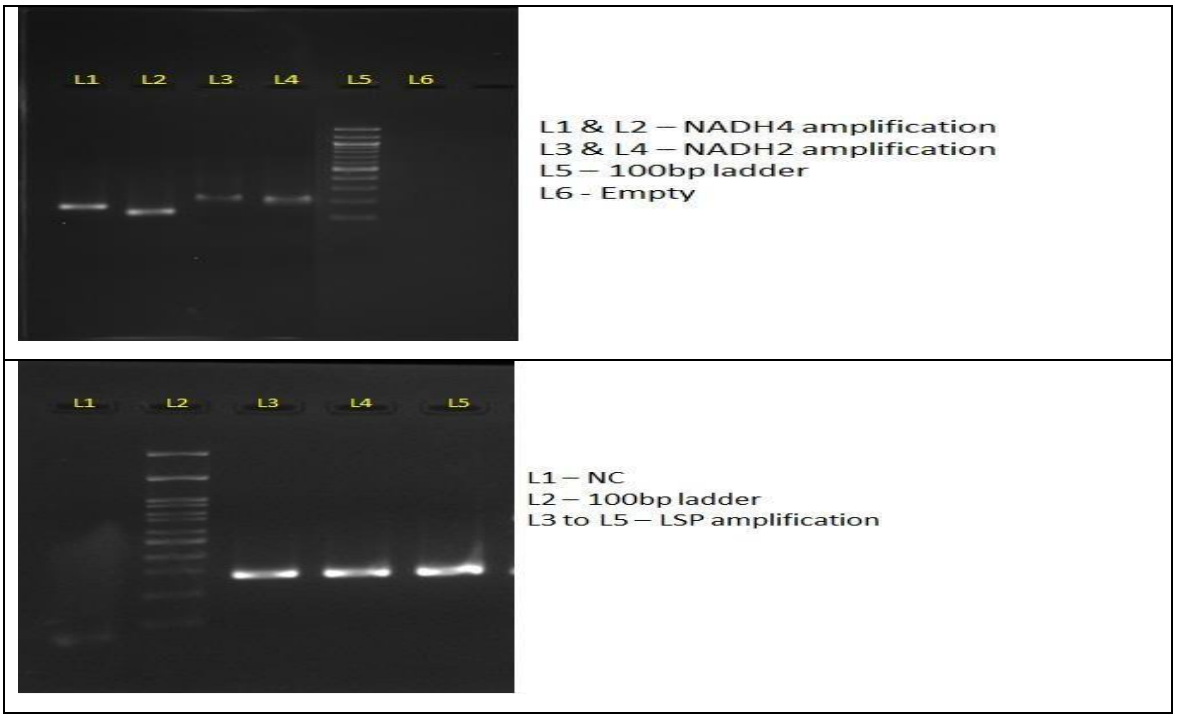


Figure 13. Gel pictures of amplified regions of scat and tissue DNA by NADH2, NADH4 and LSP primers

Table 4.3. Statistical comparison of 33 individual leopards

Number of individuals	33
Number of loci	9
Mean number of alleles per locus	7.889
Mean proportion of loci typed	0.7744
Mean expected heterozygosity	0.7475
Mean polymorphic information content (PIC)	0.6886
Combined non-exclusion probability (first parent)	0.01578986
Combined non-exclusion probability (second parent):	0.00095247
Combined non-exclusion probability (parent pair):	0.00000791
Combined non-exclusion probability (identity	1.027E-0009
Combined non-exclusion probability (sib identity):	0.00032606

.

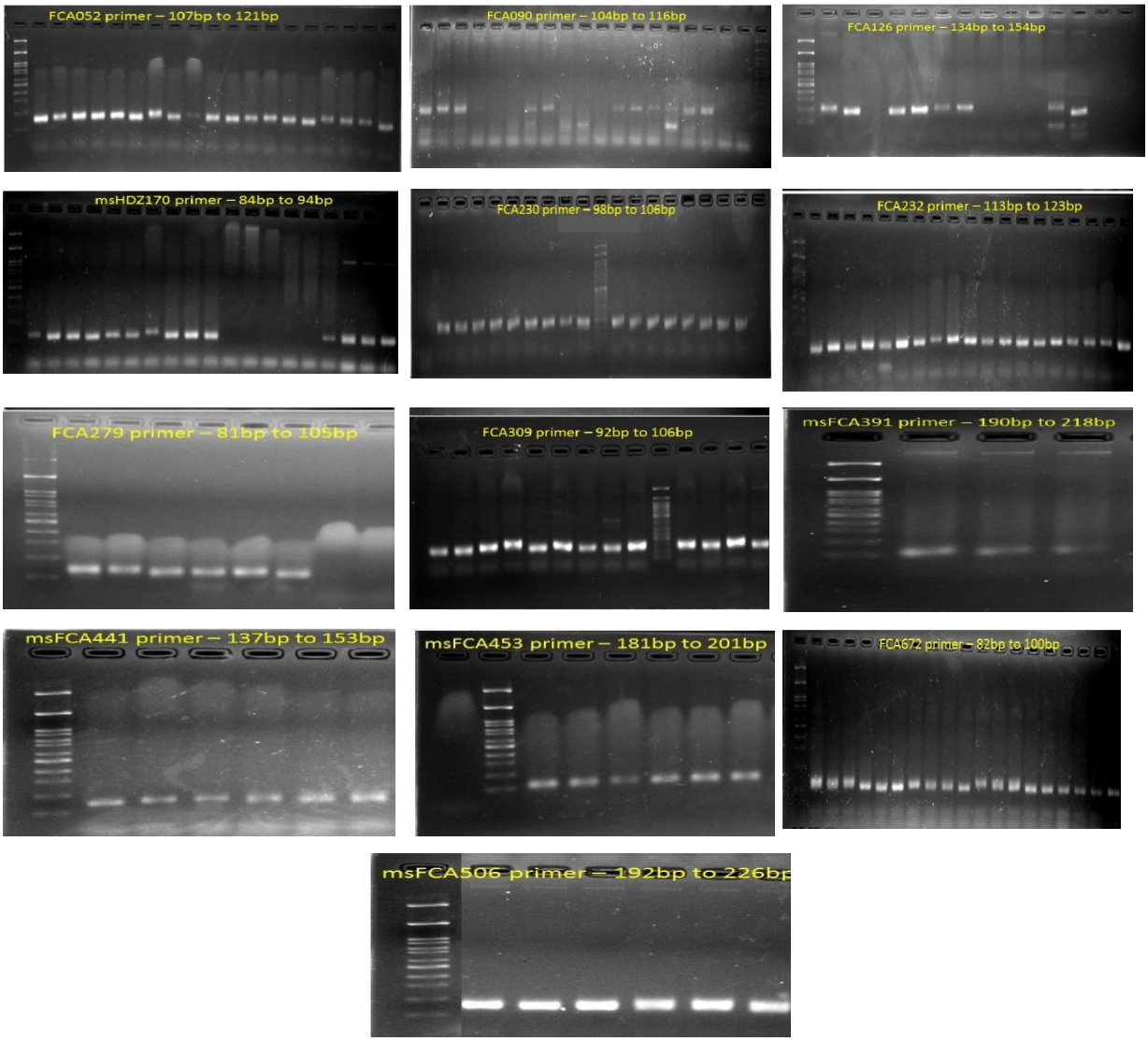


Figure 14. Microsatellite Analysis (Gel picture of base pair size of 13 microsatellite primers)

4.4 Allelic Frequency Analysis

Table 4.4. Details of Number of alleles got amplification in particular Locus and NE-Iand NE-SI

Locus	**No. of Alleles**	**NE-I**	**NE-SI**
309	4	0.288	0.541
453	5	0.136	0.432
126	14	0.038	0.334
506	13	0.044	0.343
090	8	0.059	0.358
170	4	0.180	0.466
672	7	0.162	0.457
441	11	0.062	0.362
230	5	0.147	0.441

Table 4.5. Probability of identity (individual)

Locus	NE-I	PID
309	0.288	0.288
170	0.18	0.05184
672	0.162	0.008398
230	0.147	0.001235
453	0.136	0.000168
441	0.062	1.04E-05
90	0.059	6.14E-07
506	0.044	2.7E-08
126	0.038	1.03E-09

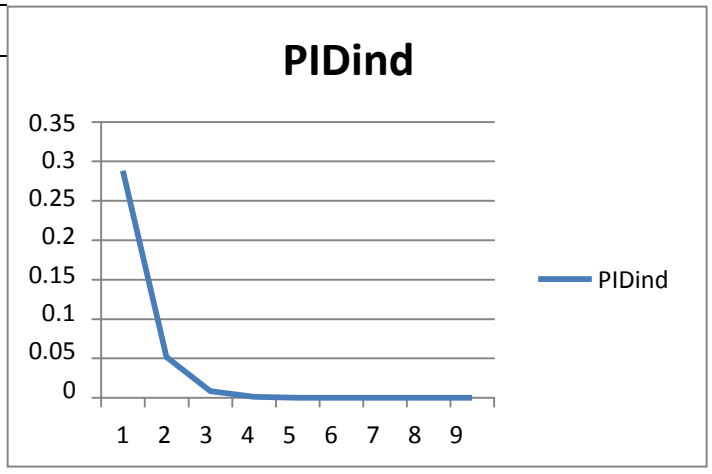


Table 4.6. Probability of identity (Sibling):

Locus	NE-SI	PID
309	0.541	0.541
170	0.466	0.252106
672	0.457	0.115212
230	0.441	0.050809
453	0.432	0.021949
441	0.362	0.007946
90	0.358	0.002845
506	0.343	0.000976
126	0.334	0.000326

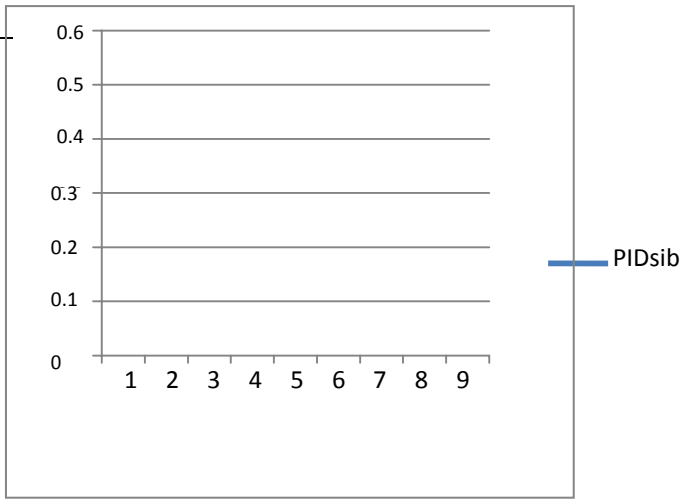
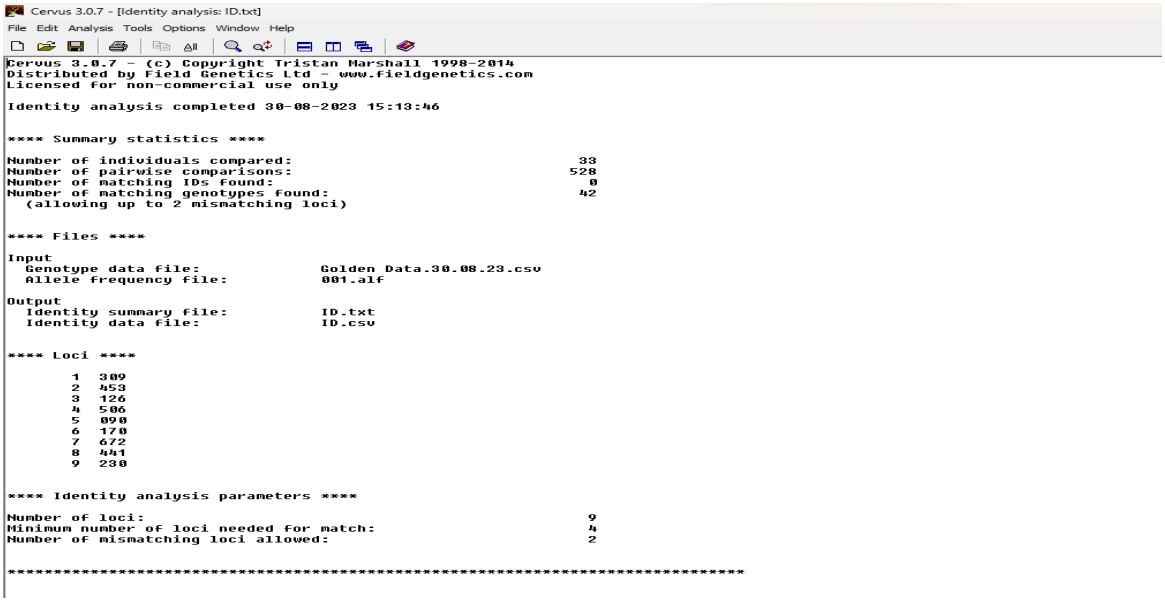
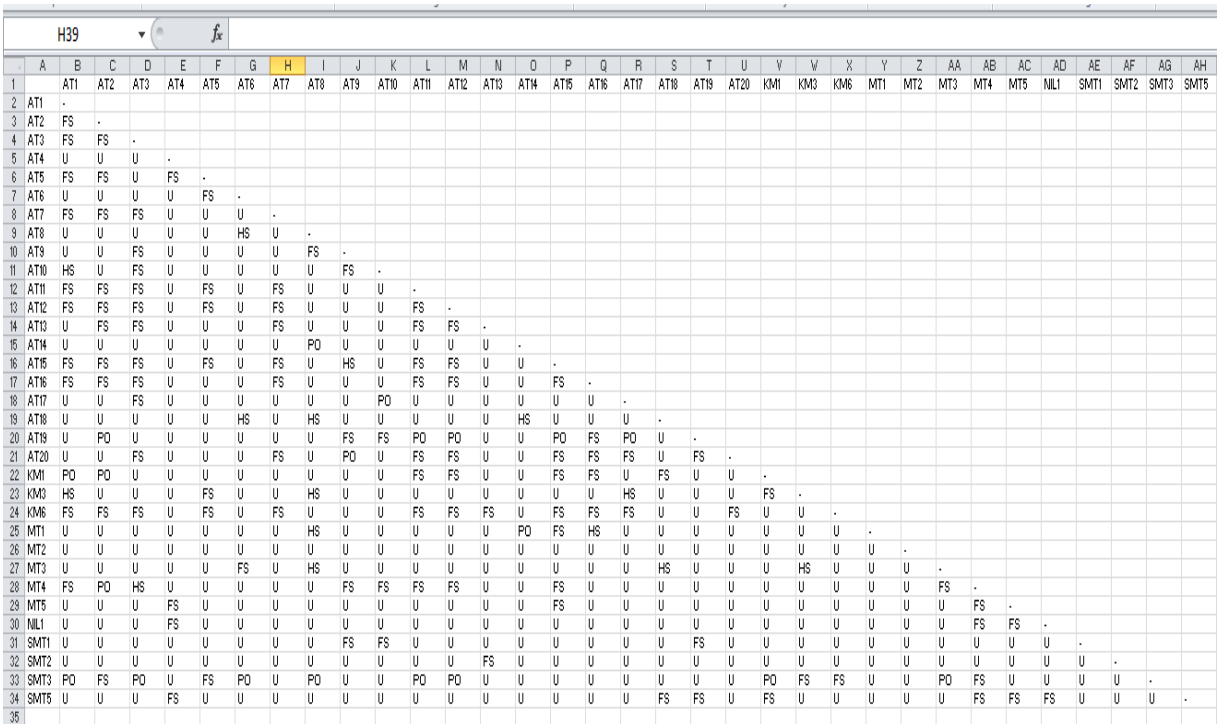


Figure 15. Identity Analysis



```
Cervus 3.0.7 - [Identity analysis: ID.txt]
File  Edit  Analysis  Tools  Options  Window  Help

Cervus 3.0.7 - (c) Copyright Tristan Marshall 1998-2014
Distributed by Field Genetics Ltd - www.fieldgenetics.com
Licensed for non-commercial use only

Identity analysis completed 30-08-2023 15:13:46

**** Summary statistics ****

Number of individuals compared:                                  33
Number of pairwise comparisons:                                 528
Number of matching IDs found:                                     0
Number of matching genotypes found:                              42
  (allowing up to 2 mismatching loci)

**** Files ****

Input
  Genotype data file:             Golden Data.30.08.23.csv
  Allele frequency file:          001.alf

Output
  Identity summary file:          ID.txt
  Identity data file:             ID.csv

**** Loci ****

       1  309
       2  453
       3  126
       4  506
       5  090
       6  170
       7  672
       8  441
       9  230

**** Identity analysis parameters ****

Number of loci:                                                   9
Minimum number of loci needed for match:                          4
Number of mismatching loci allowed:                               2

*******************************************************************************
```

Figure 16. Relationship analysis



	AT1	AT2	AT3	AT4	AT5	AT6	AT7	AT8	AT9	AT10	AT11	AT12	AT13	AT14	AT15	AT16	AT17	AT18	AT19	AT20	KM1	KM3	KM6	MT1	MT2	MT3	MT4	MT5	NIL1	SMT1	SMT2	SMT3	SMT5
AT1	.																																
AT2	FS	.																															
AT3	FS	FS	.																														
AT4	U	U	U	.																													
AT5	FS	FS	U	FS	.																												
AT6	U	U	U	U	FS	.																											
AT7	FS	FS	FS	U	U	U	.																										
AT8	U	U	U	U	U	HS	U	.																									
AT9	U	U	FS	U	U	U	U	FS	.																								
AT10	HS	U	FS	U	U	U	U	U	FS	.																							
AT11	FS	FS	FS	U	FS	U	FS	U	U	U	.																						
AT12	FS	FS	FS	U	FS	U	FS	U	U	U	FS	.																					
AT13	U	FS	FS	U	U	U	FS	U	U	U	FS	FS	.																				
AT14	U	U	U	U	U	U	U	PO	U	U	U	U	U	.																			
AT15	FS	FS	FS	U	FS	U	FS	U	HS	U	FS	FS	U	U	.																		
AT16	FS	FS	FS	U	U	U	FS	U	U	U	FS	FS	U	U	FS	.																	
AT17	U	U	FS	U	U	U	U	U	U	PO	U	U	U	U	U	U	.																
AT18	U	U	U	U	U	HS	U	HS	U	U	U	U	U	HS	U	U	U	.															
AT19	U	PO	U	U	U	U	U	U	FS	FS	PO	PO	U	U	PO	FS	PO	U	.														
AT20	U	U	FS	U	U	U	FS	U	PO	U	FS	FS	U	U	FS	FS	FS	U	FS	.													
KM1	PO	PO	U	U	U	U	U	U	U	U	FS	FS	U	U	FS	FS	U	FS	U	U	.												
KM3	HS	U	U	U	FS	U	U	HS	U	U	U	U	U	U	U	U	HS	U	U	U	FS	.											
KM6	FS	FS	FS	U	FS	U	FS	U	U	U	FS	FS	FS	U	FS	FS	FS	U	U	FS	U	U	.										
MT1	U	U	U	U	U	U	U	HS	U	U	U	U	U	PO	FS	HS	U	U	U	U	U	U	U	.									
MT2	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	.								
MT3	U	U	U	U	U	FS	U	HS	U	U	U	U	U	U	U	U	U	HS	U	U	U	HS	U	U	U	.							
MT4	FS	PO	HS	U	U	U	U	U	FS	FS	FS	FS	U	U	FS	U	U	U	U	U	U	U	U	U	U	FS	.						
MT5	U	U	U	FS	U	U	U	U	U	U	U	U	U	U	FS	U	U	U	U	U	U	U	U	U	U	U	FS	.					
NIL1	U	U	U	FS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	FS	FS	.				
SMT1	U	U	U	U	U	U	U	U	FS	FS	U	U	U	U	U	U	U	U	FS	U	U	U	U	U	U	U	U	U	U	.			
SMT2	U	U	U	U	U	U	U	U	U	U	U	U	FS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	.		
SMT3	PO	FS	PO	U	FS	PO	U	PO	U	U	PO	PO	U	U	U	U	U	U	U	U	PO	FS	FS	U	U	PO	FS	U	U	U	U	.	
SMT5	U	U	U	FS	U	U	U	U	U	U	U	U	U	U	U	U	U	FS	FS	U	FS	U	U	U	U	U	FS	FS	FS	U	U	U	.

Relationship between each pair of individuals U=Unrelated

HS = Half Sibs FS=Full Sibs

PO=Parent/Offspring

	AT1	AT2	AT3	AT4	AT5	AT6	AT7	AT8	AT9	AT10	AT11	AT12	AT13	AT14	AT15	AT16	AT17	AT18	AT19	AT20	KM1	KM3	KM6	MT1	MT2	MT3	MT4	MT5	NIL1	SMT1	SMT2	SMT3	SMT5
AT1	1																																
AT2	0.67	1																															
AT3	0.58	0.47	1																														
AT4	0.12	0.42	0.28	1																													
AT5	0.35	0.66	0.04	0.51	1																												
AT6	0.27	0.01	0	0.25	0.68	1																											
AT7	0.46	0.34	1	0.18	0	0	1																										
AT8	0	0	0.27	0	0.06	0.22	0.02	1																									
AT9	0	0	0.27	0	0	0	0.02	0.55	1																								
AT10	0.22	0.1	0.27	0	0.19	0.26	0.08	0.27	0.83	1																							
AT11	0.55	1	0.55	0.53	0.29	0.11	0.55	0	0	0	1																						
AT12	0.55	1	0.43	0.53	0.29	0.11	0.55	0	0	0	1	1																					
AT13	0.14	0.42	0.46	0.04	0	0	0.46	0	0	0	0.6	0.65	1																				
AT14	0	0	0.01	0	0	0.14	0	0.5	0.08	0.13	0.01	0.01	0	1																			
AT15	0.78	0.55	0.74	0.29	0.58	0.11	0.58	0.18	0.18	0	0.84	0.84	0.19	0	1																		
AT16	0.63	0.55	0.46	0.19	0.19	0.01	0.46	0	0	0	0.55	0.62	0.22	0	0.88	1																	
AT17	0.06	0	0.29	0	0	0	0.22	0	0.12	0.44	0	0	0	0	0.06	0.06	1																
AT18	0	0	0	0.02	0	0.23	0	0.14	0	0	0.18	0.31	0.02	0.14	0	0.02	0.15	1															
AT19	0.01	0.5	0	0.34	0.34	0.47	0	0	0.42	0.42	0.5	0.58	0.04	0	0.42	0.58	0.44	0.38	1														
AT20	0.3	0.14	0.79	0	0	0	0.63	0.02	0.41	0.08	0.33	0.33	0.29	0	0.52	0.36	0.43	0.22	0.39	1													
KM1	0.57	0.57	0.14	0	0	0	0.14	0.03	0	0	0.59	0.68	0.26	0	0.38	0.49	0	0.39	0.18	0.14	1												
KM3	0.14	0.05	0	0	0.3	0.12	0	0.21	0	0	0	0	0	0	0	0	0.23	0	0	0	0.66	1											
KM6	0.58	0.57	0.79	0.32	0.32	0	0.79	0.02	0.02	0.08	0.47	0.34	0.3	0	0.58	0.46	0.22	0	0	0.58	0.09	0	1										
MT1	0	0	0	0	0	0	0	0.33	0.37	0	0.15	0.5	0	0.5	0.57	0.31	0	0.08	0.25	0.08	0.09	0	0	1									
MT2	0	0	0	0	0	0.14	0	0	0	0	0.01	0	0	0.12	0	0	0	0	0	0	0	0	0.06	0	1								
MT3	0.29	0.01	0	0	0.11	0.6	0	0.16	0.08	0.08	0.01	0	0	0.08	0.11	0	0	0.26	0	0	0	0.19	0	0	0	1							
MT4	0.49	0.57	0.28	0.42	0.11	0.25	0.25	0	0.34	0.34	0.63	0.42	0	0.46	0.5	0.11	0	0.18	0.11	0.25	0.02	0.17	0	0	0	0.88	1						
MT5	0.34	0.11	0.34	0.57	0.01	0.11	0.34	0	0	0	0.27	0.27	0	0.01	0.47	0.34	0.1	0.18	0.11	0.34	0.09	0	0.23	0.15	0	0.01	0.79	1					
NIL1	0	0	0.1	0.6	0	0	0.01	0	0.13	0.21	0.1	0.1	0	0.33	0	0	0	0	0	0.01	0	0	0	0	0	0.21	1	0.5	1				
SMT1	0	0.12	0	0.1	0.1	0.15	0	0	0.4	0.4	0	0	0	0.19	0	0	0.11	0	0.5	0	0	0.11	0	0	0	0	0	0.16	0	1			
SMT2	0	0	0	0.2	0.2	0.1	0	0	0	0	0.01	0.2	0.46	0	0	0.11	0	0	0.19	0	0	0	0	0	0	0	0	0	0	0.06	1		
SMT3	0.5	0.6	0.5	0.31	1	0.5	0	0.5	0	0	0.5	0.5	0	0	0	0	0	0	0	0	0.5	0.65	0.6	0	0	0.5	1	0	0	0	0	1	
SMT5	0	0	0.01	0.6	0	0	0.01	0	0	0	0.1	0.27	0.02	0.01	0	0	0.15	0.61	0.48	0.36	0.42	0	0	0.25	0	0	0.25	0.5	0.6	0	0	0	1

Figure 17. Maximum likelihood relatedness analysis

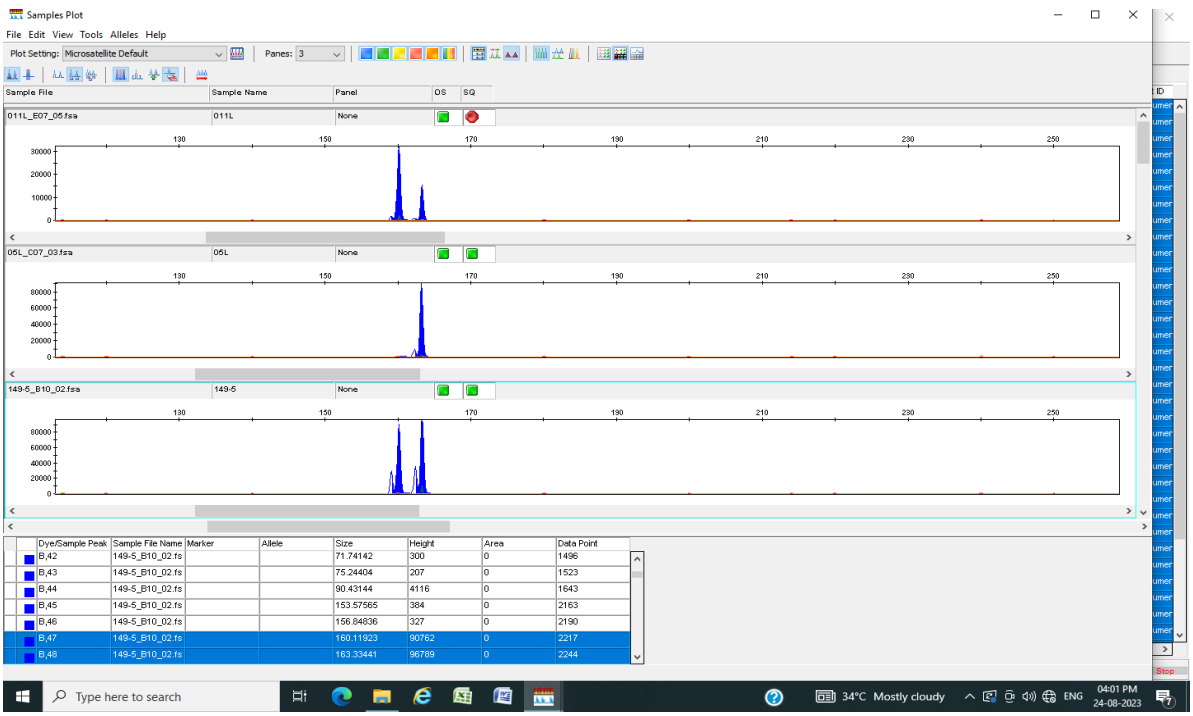


Figure 18. Sex Identification With ZNF (Zinc-finger)primer (Male- 163 & 166 and Female-166)

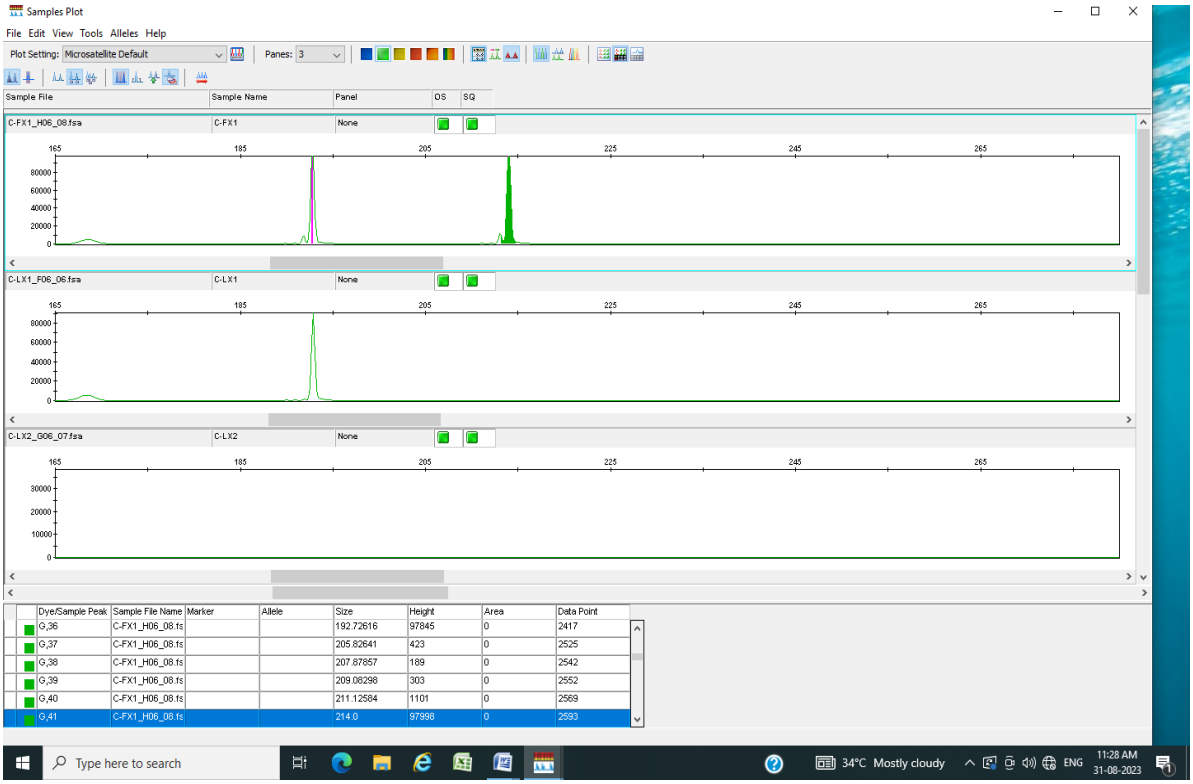


Figure 19. Sex Identification With AML (Amelogenin) primerMale-194 & 204andFemale-204)

Table 4.7. Gender Identification of collected scat samples

Tiger Reserve	No. of Male	No. of Female
MTR	4	1
SMTR	2	2
KMTR	3	3
ATR	14	6

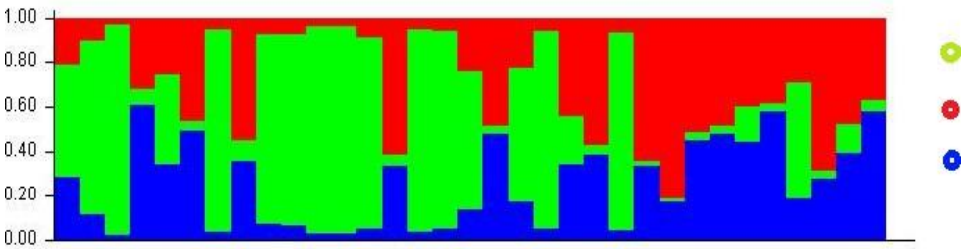


Figure 20. Structure analysis bar plot represents the number of populations with co-occurrence of healthy gene-flow

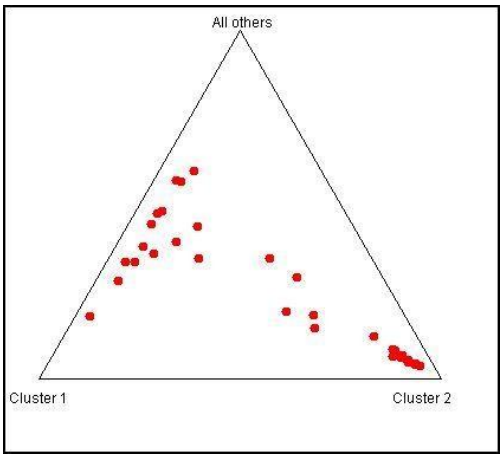


Figure 21. Triangle plot indicates the clusters of population in a given sample

5.DISCUSSION

The detection and characterization of population structure is one of the cornerstones of modern population genetics and wildlife forensics with an intention to conserve the prestigious endangered species. The present study utilized microsatellite markers to investigate individual and geographic identification of leopards (*Panthera pardus*) in a specific region. The results provide valuable insights into the population structure, genetic diversity, and potential conservation implications for this elusive and charismatic species. In this discussion, we will delve into the key findings, their implications, and the broader significance of the study.

The assessment of genetic diversity using 13 STR primers provided insights into the allelic richness and heterozygosity of the sampled leopard population. The choice of STR markers is crucial, as their high mutation rates allow for a finer resolution of genetic diversity. The observed genetic diversity metrics offer a baseline against which future genetic changes can be measured. Comparisons with historical or neighbouring populations could reveal shifts in genetic diversity and potential threats to the population's genetic health (Manier, 2005)

The analysis of microsatellite data unveiled the population structure and genetic differentiation among leopards across the study area (MTR, SMTR, ATR, KMTR). Distinct genetic clusters can indicate barriers to gene flow or connectivity between subpopulations. This knowledge is vital for designing conservation plans that prioritize the preservation of genetic diversity, especially when considering the potential impacts of habitat fragmentation and human-induced landscape changes.

Photographic surveys are also logistically challenging in landscape studies as they require equipment, skilled personnel, and intensive effort in low animal density areas, particularly outside protected zones. The molecular tools developed in this study thus provide certain advantages over earlier approaches used in carnivore studies (Mondol *et al.,* 2014). Thus,

the field-collected scat samples from five tiger reserves of Tamil Nadu have been subjected to PCR-based species, sex and individual identification.

Faecal samples from the field conditions could potentially result in highly variable DNA quality and quantity, resulting in possible errors in the genotype (Mondol *et al.,* 2009). By amplifying the panel of 13 microsatellite loci for 38 field-collected PCR-confirmed leopard samples, the success rate of amplification was obtained as 87% for individual and geographical identification and 100% success rate for sex identification of leopards using STR. Out of 13 microsatellite loci, the study has been further carried out with 9 microsatellite markers, which showed greater success.

The use of 9 different micro satellite loci has demonstrated a robust method for identifying individual leopards. The multi-locus approach enhances the accuracy and reliability of individual identification by examining genetic variation across multiple loci. This accuracy is pivotal for studies involving elusive and solitary species like leopards, where direct observations are challenging. The results underscore the efficacy of microsatellite analysis in distinguishing between closely related individuals, which is essential for estimating population size, monitoring survival rates, and understanding behaviour.

PID analysis for individuals and siblings (PID ind and PID sib) suggests i) a panel of five microsatellite markers for individual identification and ii) seven microsatellite markers for siblings identification. Allelic frequency analysis reveals the highly polymorphic loci, which is crucial for individual and sibling identification (126, 506, and 441 primers) (Table 4.4).

A comprehensive identity analysis for pairwise comparison was conducted using genetic data to assess the individual uniqueness and relatedness of leopards (*Panthera pardus*) with in a given sample (n=33).

Relationship analysis reveals parental, full sibling, half sibling and unrelated species. Leopard samples obtained from Mudumalai tiger reserve

(MTR) showed distinct variation when compared with Anamalai (ATR), Srivilliputhur-Megamalai (SMTR) and Kalakad-Mundandurai Tiger reserves (KMTR). But showed high relatedness among ATR, SMTR and KMTR.

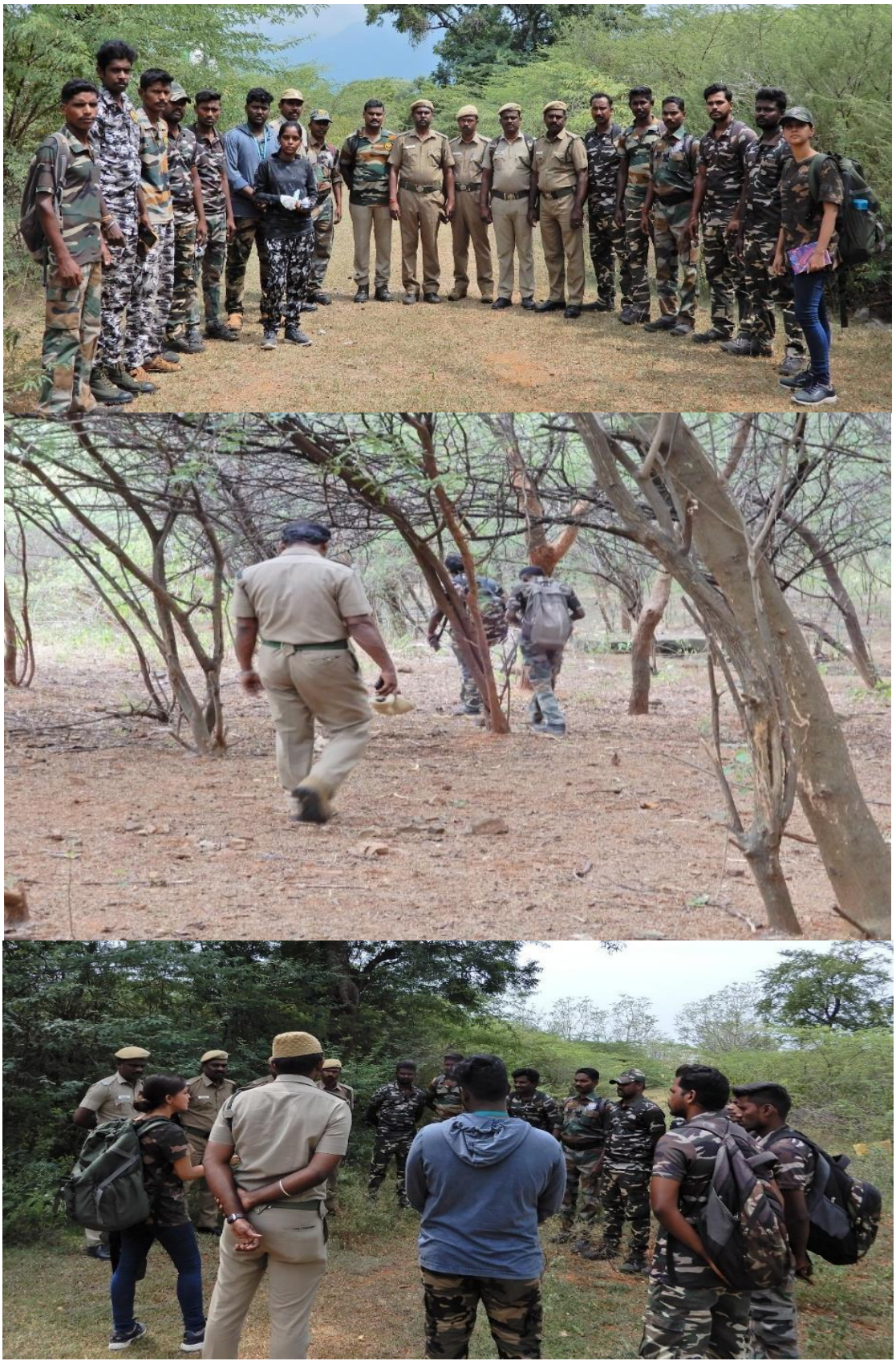
In the context of the current study, a maximum likelihood relationship analysis was employed to investigate the genetic relatedness and kinship among individuals within a leopard (*Panthera pardus*) population. This sophisticated approach utilizes genetic data to probabilistically infer the most likely familial relationships among individuals. In this discussion, we will delve into the study's utilization of maximum likelihood analysis, its implications, and its significance in enhancing our understanding of leopard population dynamics and conservation.

Geolocation assessment was done using Structure analysis which is designed to detect underlying patterns of genetic structure within a population that utilizes genetic data to infer the genetic structure and identify potential subpopulations or clusters of individuals with shared ancestry. This approach aids in understanding the spatial distribution of genetic diversity and has important implications for conservation and management strategies. In this discussion, we will explore the study's use of structure analysis, its findings, and its relevance for leopard conservation. Our finding shows that within the given sample, we can classify three diverse populations that are related to each other, showing healthy gene flow between the sample collected area. Triangle plots offer a visual representation of genetic diversity and relationships within a population. Each corner of the triangle corresponds to a hypothetical pure genetic cluster, and individual data points are plotted within the triangle based on their estimated genetic composition from those clusters. The position of each individual in the triangle indicates the proportion of genetic contributions from the different clusters. In our study we obtained three two distinct clusters and an inter-mediate cluster which represents the genetic diversity between the population.

Some Fieldwork Photos on scat sample collection and training programmes

Training conducted in Hassanur range, Sathyamangalam Division

Training conducted in Thalavadi range, Satyamangalam Division



Training Conducted in Bhavanisagar range, Satyamangalam

Training conducted in Kargudi range, Mudumalai Division

Training conducted at Oveli range, Gudalur Division

Training conducted at Singara beat, Masinagudi range

Field visit and training at Sigur beat, Masinagudi Range

Training programme for field staff on leopard scat sample collection for DNA analysis at Theppakadu auditorium

Tiger scat **Leopard scat**

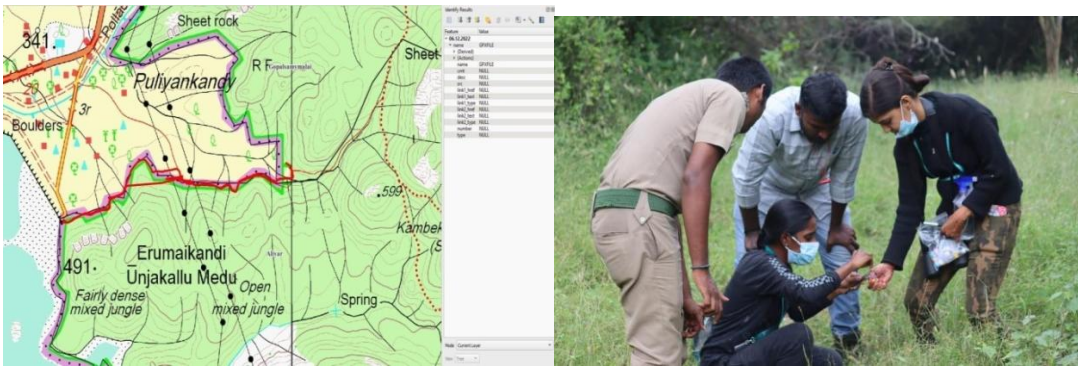
Field work for scat sample collection at Singara range

TIGER PUGMARK

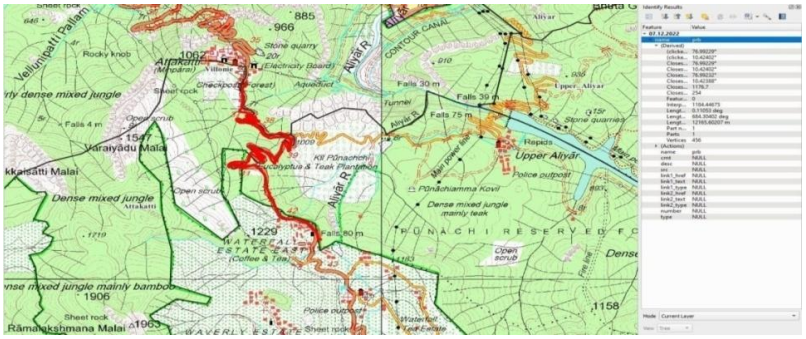
LEOPARD PUGMARK

Training conducted at Doddabetta range, Nilgiri division

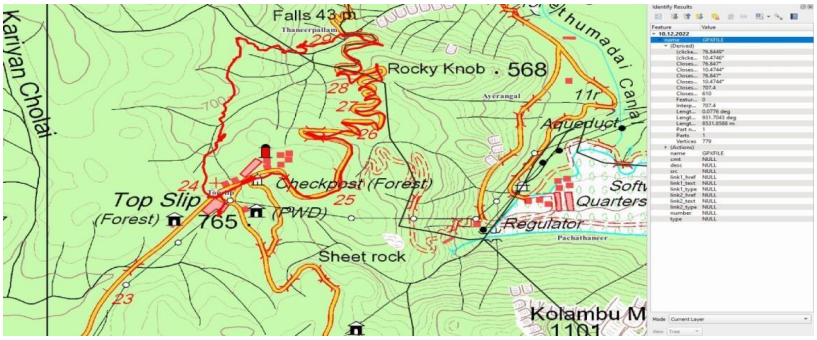
Training on leopard scat sample collection in Pollachi and Tiruppur division



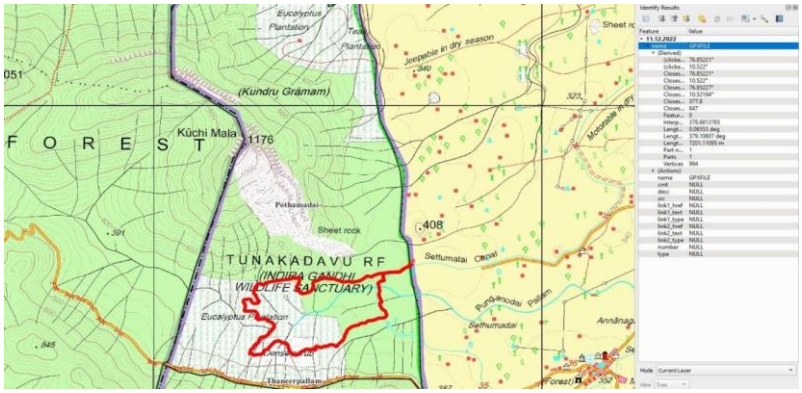
Aliyar track map and collection of scat samples at Aliyar beat



Attakatti track map covered during scat collection



Track map covered during scat collection at Topslip

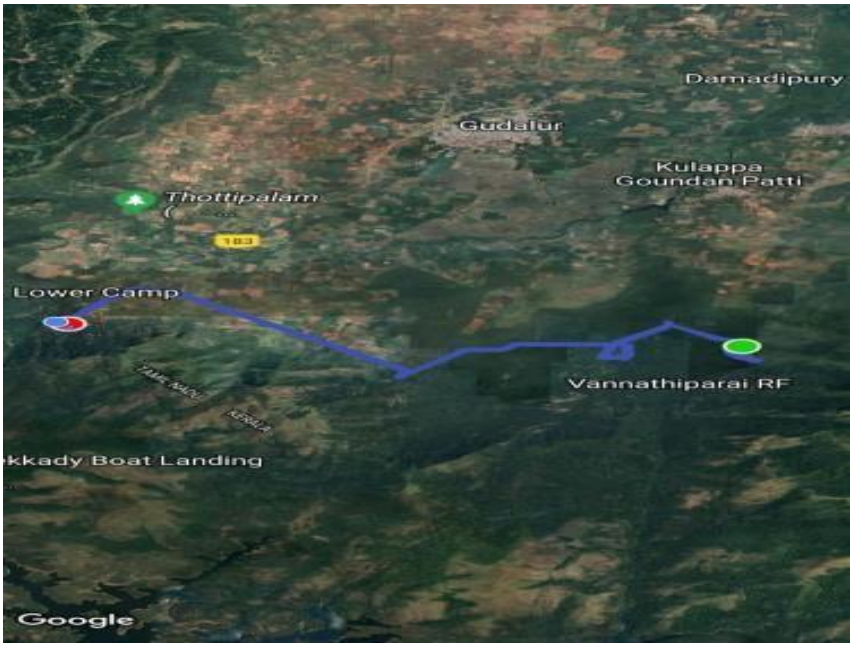


Sethumadai track map covered during scat collection

Track maps showing areas covered during fieldwork for scat sample collection in Aliyar, Attakatti, Topslip and Sethumadai

Training and leopard scat sample collection at Srivilliputhur Division

Training and leopard scat sample collection at Megamalai Division



Track map of the visited field at Megamalai division

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