DNA Techniques in Wildlife Forensics (Animals):

Standard Operating Procedures (SOP)

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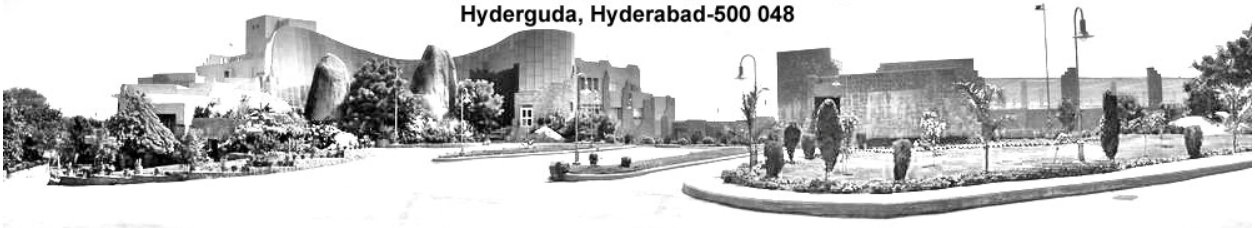

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FOREWORD

Species are poached and illegally harvested at increasingly unsustainable levels. Wildlife crime has become the fourth most lucrative illegal business after narcotics, human trafficking, and weapons. The cost of such environmental crime to developing countries is estimated to be more than $70 billion a year, according to the World Bank in 2014. As wildlife crime increases, it results in environmental degradation. This adversely affects ecosystem services, which in turn affects the livelihoods, fuel and food for local communities that are dependent on them.

In response to the growing crisis, CCMB-LaCONES has been providing services to identify species, and individual animals involved in wildlife crimes. This service is heavily subscribed by different user agencies. While the reporting of crimes and their investigations are growing rapidly, the facilities that provide diagnostic services have not correspondingly increased. Certainly, there is scope to scale up such diagnostic services so that they are accessible and affordable to all users. This SOP is meant to trigger interest among the various users to adopt these procedures and effectively solve wildlife crime cases.

- Rakesh Mishra
Director, CSIR-CCMB

The illegal wildlife trade is a global threat. The problem is particularly acute in developing countries, where there are iconic species, such as tiger, Asian elephant, one-horned rhinoceros. Several new wildlife items are included in illegal wildlife trade regularly. Pangolin scales, red sand boa, giant gecko and monitor lizards are being poached to extinction for their body parts. These items are unrecognizable by sight, and identification of the species involved from such material is extremely challenging. The protocol outlined here is the outcome of decade long effort of testing, standardising and successful application of DNA-based technologies in wildlife forensic diagnosis by the scientists. If this SOP is followed by interested parties, several wildlife offenses could be resolved in a timely manner.

- Karthikeyan Vasudevan
Scientist-In-Charge, CCMB-LaCONES

PREFACE

The primary objective of a wildlife forensic investigation is to identify the species from seized biological samples, assess relatedness if multiple individuals are seized and repatriation to their original environment if required. The use of forensic science ensures enhanced specificity in counter-measures against wildlife crimes by law enforcement agencies. In accordance, DNA-based analysis in forensics has brought about substantial precision in species identification and evaluation of relatedness and it is thus becoming increasingly preferred as courtroom evidence.

The first step towards successful identification of species is the systematic collection and storage of specimen for DNA analysis in the lab. Each type of seized biological sample requires specific method for collection, handling, and forwarding to the lab for DNA analysis. Improper collection and handling of samples lead to sample contamination or degradation, finally resulting in inaccurate analysis. Use of techniques that are not validated and have no reference data for comparison lead to incompatible reporting on cases. The lack of standard operation procedures in forensic investigation and diagnostics limits the success rate of conviction of wildlife crimes. Therefore, a Standard Operating Procedure (SOP) of detailed, accurate and universally accepted protocols is a necessary requirement for any Wildlife Forensics Diagnostics laboratory.

In order to address this necessity, we have brought out a SOP that provides details of the procedures and protocols; and adapted them to suit our field conditions. It would enhance our efficiency in sample collection, sample storage, transport of seized biological materials to the Lab, DNA isolation, amplification and data analysis for species identification, individual identification, relatedness, and sex identification. We hope this SOP will serve as a guidance document for all the users involved in DNA-based wildlife forensics.

We sincerely thank Siuli Mitra, A. Sreenivas, Harika Katkam, Drishti Parmar, Vaishnavi Kunteepuram, Andrea Luis, Noopur Modi and Rajyalakshmi for their contributions.

- Ajay Gaur
- P. Anuradha Reddy

CONTENTS

LIST OF ABBREVIATIONS

µl – Microliters
µM – Micro molar
A – Absorbance
BLAST – Basic Local Alignment Search Tool
bp – Base Pair
BSA – Bovine Serum Albumin
BSS – Balance Salt Solution
CCD - Charged Couple Device
CCMB– Centre for Cellular and Molecular Biology
cm – Centimeter
CSIR– Council for Scientific and Industrial Research
Cytb – Cytochrome b
dATP – deoxy Adenosine triphosphate
DC – Direct current
dCTP – deoxy Cytosine triphosphate
ddNTP – dideoxy nucleotide tri phosphate
dGTP – deoxy Guanosine triphosphate
DMSO – Dimethyl Sulfoxide
DNA – Deoxy ribonucleic acid
dNTP – deoxy nucleotide triphosphate
DTT–Dithiotreitol
dTTP – deoxy Thymidine triphosphate

DW– Deionised water
EDTA – Ethylene Diamine Tetra Acetate
EtBr – Ethidium Bromide
FASTA – Fast Alignment
g – Gram
K_3EDTA – Potassium salt of ethylene diamine tetra acetate
KCl – Potassium chloride
LaCONES – Laboratory for the Conservation of Endangered Species
M – Molar
Mcb – Mitochondrial cytochrome b
mg – Milligram
$MgCl_2$ – Magnesium Chloride
ml – Milliliter
mM – Millimolar
mtDNA – Mitochondrial DNA
Na_2HPO_4 – Sodium hypophosphate
NaCl – Sodium chloride
$NaClO_4$ – Sodium perchlorate

NaOH – Sodium hydroxide
NCBI – National Centre for Biotechnology Information
ng – Nanogram
NH_2PO_4 – Ammonium phosphate
nm – Nanometer
PBS – Phosphate Buffer Saline
PCI – Phenol Chloroform Isoamyl alcohol
PCR – Polymerase Chain Reaction
pg – Pico gram
pM- Pico moles
RBC – Red Blood Cells
rpm –Rotations per minute
RT qPCR – Real Time Quantitative Polymerase Chain Reaction
SDS–Sodium Dodecyl Sulphate
STR – Short Tandem Repeat
TAE –Tris Acetic Acid EDTA
TE – Tris EDTA
TES – Tris EDTA Saline
UV – Ultra Violet

V- Volt
WBC – White Blood Cells

1

COLLECTION, PRESERVATION AND TRANSPORTATION OF BIOLOGICAL SAMPLES TO THE DNA LABORATORY

INTRODUCTION

Monitoring of illegal wildlife trade primarily requires species, individual and sex identification from animal body parts and products seized at locations differing from the area in which the investigator is working in. Identification using morphological characters often lacks the required precision and fails to produce meaningful evidence for forensic investigation. In comparison, modern molecular approaches that evaluate DNA (molecular) signatures for identification have greater utility. DNA can be extracted from highly processed and degraded wildlife products such as, cooked and dried meat, claws on tanned hides, dried shark fins, egg shells, animal hair, bones, ivory, horns, turtle shell, feathers and fish scales and even from baggage used for transporting wildlife products illegally. The scope of DNA-based analysis in forensic investigations extends from application in identification of unknown species to assessing relatedness among individuals and populations. Forensic scientists take advantage of the different classes of genetic variation to circumvent the challenges posed by forensic cases like identification of species of an unknown sample of charred bones in a courtyard or to determine the geographical origin of animal products like hide or ivory ceased at an airport terminal. Wildlife forensics has been developed as a major tool in keeping a check over wildlife crimes.

CCMB-LaCONES is one of the leading laboratories in the country recognized to do DNA-based wildlife forensics. Here, DNA-based species identification, individual identification, sexing, and geographic assignment of wildlife that being illegally trafficked are being carried out since 2000. It is a major on-going activity at LaCONES. The technique developed and patented by CCMB–LaCONES allows identification of a biological specimen of unknown origin, and delineates its utilityit to the level of family, genus and species. Many biological specimens are received from all over the country forwarded by state forest, judiciary, police, and custom departments. From the year 2000 till present, more than 19500 wildlife crime cases have been received at LACONES, which includes more than 43500 biological samples such as meat, cooked meat, bones, faeces, dried, and chemically treated skin, ivory, hair, nails, snake venom, blood stains, horns, and antlers.

The preliminary stages in DNA-based analysis is the collection, preservation and storage of biological specimen for laboratory analysis. Mistakes in collection and handling of samples lead to sample contamination or degradation eventually plaguing the precision of analysis. Cross-contamination of samples may also occur when different biological samples are handled

at the same time which, in turn, is difficult to avoid given the large number of cases, and comparatively less infrastructure. This chapter enlists Standard Operations Procedure (SOP) for collection and preservation of various tissues as forensic evidence. The chapter also deals with how to avoid cross-contamination of samples. These practices could improve yield of good quality DNA for subsequent analyses.

Blood

Description of specimen: Blood drawn from an animal carcass or live animal

Requirements:

- Sterile gloves
- Hand sanitizers
- Disposable syringes
- K3EDTA-coated vials
- Cotton
- Absolute alcohol/ rectified spirit
- Ice-box
- Marker for labelling

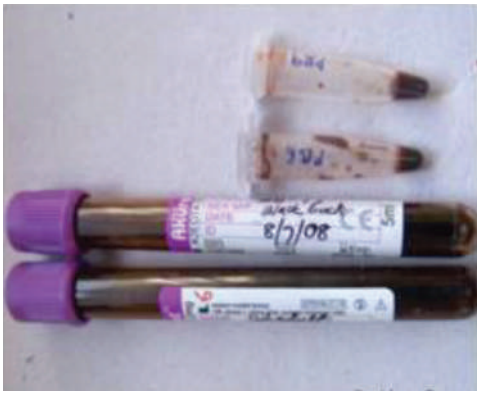


Figure 1. K3EDTA vacutainers and tubes with blood samples

Instructions for collection:

1. Anesthetize the animal before drawing blood in case the animal is alive. This step should be done in the presence of a qualified veterinarian and only a trained technician/phlebotomist should be allowed to draw blood.
2. Transfer the blood immediately to a K3EDTA-coated vial (commercially available at medical stores) and label the tube specifying name, age, sex of the animal and date and location of collection (Fig 1).

Storage and transport:

1. Store the sample vial in an ice-box till it is transferred to a refrigerator.
2. For transferring to a DNA laboratory for analysis, it should be properly packed in a compartment that will ensure a low storage temperature and prevent any mechanical damage during transport.

Precautions:

1. Administration of anaesthesia and drawing of blood MUST be carried out in the presence ofqualified a veterinarian./phlebotomist.
2. Forceful restraint of animal should always be avoided.

3. Gloves and masks should be worn by the technician and other personnel.
4. Personnel involved in the collection must sanitize their hands before and after the collection.

Blood stains

Description of specimen: Blood- stained material for example soil, plant parts, clothes, knife or an axe (Fig 2).

Requirements:

- Sterile gloves
- Hand sanitizers
- Surgical mask
- Zip lock pouch/ Petri dish
- Spatula
- Plastic vial (in case of soil or dried blood scraped from knife or axe)

Instructions for collection:

1. Place a few particles of blood- stained soil matter in a clean and dry test tube.
2. Wrap the blood-stained plants parts or soil in a clean plastic zip lock bag/pouchand seal well for storage.
3. Wrap the blood-stained cloth in a clean plastic zip lock pouch and seal well for storage.
4. Scrape the blood stains from the knife or axe (NOT VIGOROUSLY) to remove the dried stain. Collect the scrapings in a clean and dry test tube.

Storage and transport:

The sample tubes can be stored in a dry box containing an ice-pack for transport.

Precautions:

Personnel should always wear gloves to prevent contamination by touching the sample as it is very small in amount in case of blood stains.

(A)

(B)

(C)

Figure 2. Blood stained (A) sickle and knives, (B) soil and (C) leaves

Feces (or scat)

Description of specimen: Feces (or scat)(Fig 3)

Figure3. Fresh Scats of a carnivore

Requirements:

- Sterile gloves
- Hand sanitizers
- Surgical mask
- Sterilized collection spoon
- Silica gel beads
- Zip lock pouch / Petri dish
- 70% ethanol
- 50 ml collection tubes

Instructions for collection:

1. Collect faeces in one of the containers with a sterilized and dry spatula. The faeces should not be older than 72 hours.
2. Place the sample inside a clean and dry zip lock pouch consisting of silica gel beads which will absorb moisture to prevent microbial contamination (Fig 4).
3. Alternatively collect the sample in 50 ml collection tube and submerge sample in 70% ethanol.

Figure 4. Scat sample and silica beads (blue turned pink) sealed in a ziplock pouch

Storage and transport:

The zip lock cover or collection tube can be placed in an ice box for transport or kept at 4°C for short term storage in the laboratory.

Precautions:

1. The feces scat should not be older than 72 hours.
2. Care should be taken to prevent moisture and consequent microbial contamination by using silica beads or alcohol for storage.

Hair/Feathers

Description of specimen: Animal hair or bird feathers (Fig. 5).

Requirements:

- Forceps
- Zip lock pouch/Petri dish

Instructions for collection :

1. Pluck, and DO NOT CUT, hair or feathers with forceps ensuring that the root of the hair shaft or calamus of the feather stays intact.
2. Transfer the sample into a clean and dry plastic zip lock pouch (or Petri dish) and label appropriately

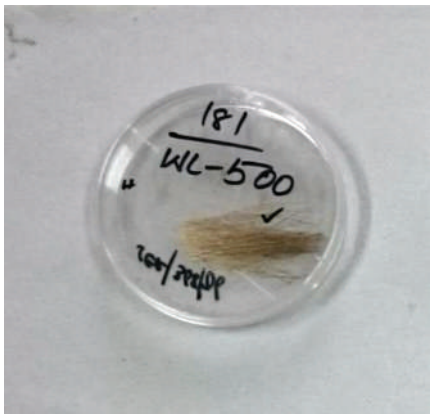


Figure 5. Hair sample placed in a Petri dish

Storage and transport :

The plastic pouch containing hair samples does not require low storage temperature and can be kept in a simple box labeled for transferring to the DNA laboratory.

Precautions:

1. Hair or feather samples SHOULD NOT BE CUT but plucked with the root intact as DNA can only be isolated from the tissues located in the root.
2. Avoid touching the basal part of hair or feathers.

Horn/Ivory/Bone

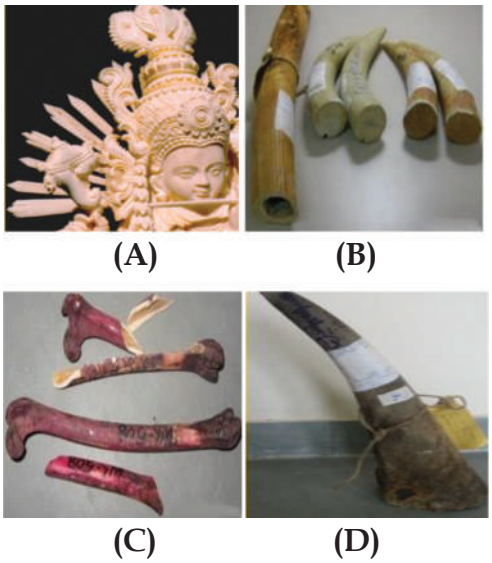
Description of specimen: Ivory/charred/ Horn bones of an animal (Fig 6).

Requirements:

- Driller
- Filer
- Petri dish
- Zip lock pouch/Petri dish

Instructions for collection:

1. Take the piece of bone (or horn or ivory) and wipe it with a dry cloth to remove any external impurities.
2. Wipe a filer with alcohol to sterilize it and use it to cut small pieces of the hard tissue. Place the pieces obtained in a Petri dish or a zip lock pouch.



(A) **(B)** **(C)** **(D)**

Figure 6. (A) Ivory idol, (B) Ivory (extreme left sample is an original and the other samples are fake), (C) Charred animal bones and (D) Horn

Storage and transport:

The sample can be wrapped in a paper, white cloth or aluminum foil and stored at room temperature (Fig 7) for transporting to the laboratory. Bones with soft tissue should be stored at 4ºC.

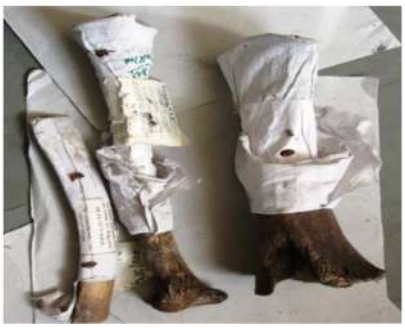


Figure 7. Bone wrapped in white cloth

Precautions:

1. The personnel handling the tissue should always wear gloves to avoid human contamination.
2. The filer should be cleaned with alcohol before use.
3. In case of multiple samples, the filer should be cleaned thoroughly between samples.

Meat

Description of specimen: Cooked, partially cooked or raw meat (Fig 8)

Requirements:

- Sterile blade
- Petri dish
- Salt
- Zip lock pouch

Instructions for collection:

1. Cut a slice of flesh with a sterile blade.
2. Place the slice in a jar containing salt or ethanol as a preservative.

Storage and transport:

The container with the flesh sample should be stored in an ice box for a short period or in a 4°C refrigerator for a longer period.

(A)

(B)

Figure 8. Meat samples in (A) Jar with salt and (B) alcohol

Precautions:

1. Personnel must always wear gloves.
2. The blade used for slicing should be wiped with alcohol before and after use.
3. In case of multiple samples, the blade should be cleaned thoroughly between samples.

Skin

Description of specimen: Skin(Fig 9)

Requirements:

- Pair of scissors or blade
- Zip lock pouch/Petri dish

Instructions for collection:

1. Clean the scissors or blade with alcohol for complete sterilization.
2. Cut approximately 5 cm^2 of the skin or hide
3. Place the skin piece in a clean and dry zip lock cover or Petri dish (Fig9B).

Storage and/ transport:
The sample can be kept at room temperature.

Precautions:

1. Scissors used for cutting the skin piece should be properly sterilized.
2. Personnel must wear gloves while handling the sample to avoid contamination.

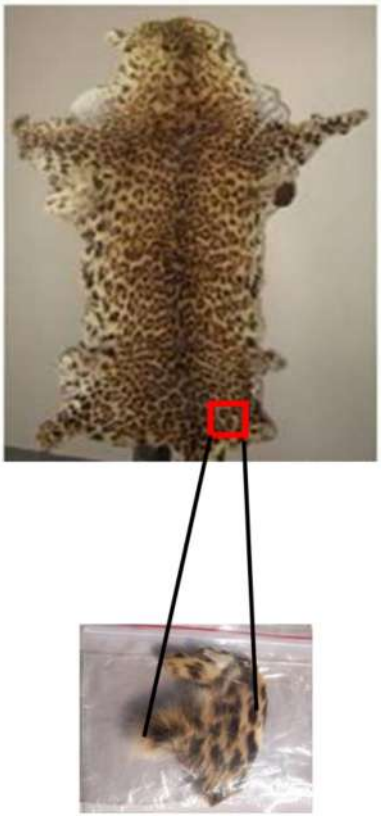


Figure 9. (A)Skin of leopard and a (B)small sample taken from it and placed in a zip lock cover

Table 1. Summary of methods for short-term preservation and storage for different types of tissues

S No.	Type of tissue	Preservation	Storage
1	Blood	K3EDTA	4°C
2	Blood stain	-	Room temperature
3	Scat (feces)	Silica beads/ alcohol	4°C
4	Hair/feather	-	Room temperature
5	Meat	Salt/saline/ethanol	4°C
6	Bone/ivory/horn	-	Room temperature
7	Skin	-	Room temperature

Collection and preservation of soft tissue for DNA analysis

Tissues samples can be collected and stored in different media, choice of which may depend on the availability of reagents and working conditions. Under good laboratory conditions, tissues can be collected and flash frozen in liquid nitrogen before storing at -20 ^{o} C to -70 ^{o} C. On the other hand, in field conditions, the samples (cut into small pieces) may be collected in one of the following buffers/ solutions.

- 20% NaCl-saturated DMSO
- 90-95% ethanol
- 0.9% NaCl
- TES
- PBS

Among the above, the DMSO solution provides one of the best medium. Here it may be noted that formalin which is generally used as a preferred medium for preserving samples, should be avoided when samples are intended for use in DNA analysis.

METHODS OF DNA ISOLATION FROM DIFFERENT BIOLOGICAL SPECIMENS

The extraction of genomic DNA marks the initiation of laboratory analysis in molecular genetic studies. The basic steps involved in DNA extraction are lysis of cells, protein and lipid extraction, DNA isolation and purification of isolated DNA.

DNA can be extracted from fresh or frozen whole blood, blood stains, hair, meat, bone and other biological samples by application of protocols customized to suit the tissue type. The PCI method is most widely used for DNA isolation. This method gained popularity owing to its ability to separate protein and other cellular components from nucleic acids and subsequently remove them resulting in purer DNA preparations in comparison to other known methods. Stated below are steps followed for DNA isolation by PCI method for different biological specimen.

Prerequisites for DNA isolation

Equipment

- Centrifuges
- Auto pipettes
- Water bath
- Dry bath
- -20^{o}C refrigerator
- DNA isolation bench
- PCR hood

Plastic ware

- Falcon tubes
- Eppendorf tubes
- Pipette tips
- Tube stands

Chemicals

- Sucrose
- MgCl2
- Triton X
- Tris-HCl
- Na2-EDTA
- NaCl
- Proteinase K
- SDS
- Sodium perchlorate
- KCl
- Na2HPO4
- NH2PO4
- Chloroform
- Iso-amyl alcohol
- Isopropanol
- PBS
- DW
- Tris-equilibrated phenol

Preparation of reagents used for different protocols for DNA isolation is described in Annexure I and should be referred to before starting the protocols.

Scope:

This method can be used to obtain DNA from different types of tissue, such as peripheral blood, cell lines or tissues.

DNA isolation from fresh blood with sodium per chlorate

1. To one volume of blood, add 4 volumes of reagent A in a polypropylene tube. Mix gently till the solution becomes clear.
2. Centrifuge at 2500 rpm for 5 minutes to obtain a pellet free from RBCs. Discard the supernatant containing lysed RBCs carefully.
3. Disturb the pellet thoroughly and add half the volume (as that of blood sample) of reagent B. Mix thoroughly and gently by inverting for 3-4 minutes till the solution becomes viscous.
4. Add reagent C (1/4th volume of reagent B) and mix gently for 3-4 minutes.
5. Add equal volume (as that of reagents B and C) of tris-equilibrated phenol and chloroform. Mix well and centrifuge at 2500-3000 rpm for 7-8 minutes to separate into 3 layers viz. aqueous layer, protein layer and solvent layer.
6. Transfer the aqueous layer carefully into another centrifuge tube using a broad mouth tip (Care should be taken that the protein layer is not disturbed).
7. Add equal volumes of chloroform to the supernatant and mix gently for a minute and centrifuge at 2500 rpm for 5 minutes.
8. Transfer the aqueous phase to a fresh tube.
9. Add two volumes of chilled absolute alcohol and mix gently to precipitate the DNA.
10. Spool out the DNA lump in a fresh Eppendorf tube and decant alcohol.
11. Wash the DNA twice with 70% ethanol and give a short spin to remove alcohol.
12. Dry the pellet properly in a dry bath and ensure that whole alcohol is dried off.
13. Dissolve the pellet in 50-100µl of TE buffer.
14. Incubate at 55^{o}C for 3 hours to enhance the dissolution.
15. Store the DNA samples at -20^{o}C.

Soft Tissues (meat, cooked meat, flesh, dried meat and skin pieces)

1. Cut tissues into small pieces with a sterile scalpel and separate out any adherent adipose tissues from it. Put the pieces in 1X PBS solution and wash overnight to remove external contaminants.
2. Mince the small pieces using a sterilized blade and add about 500 mg of tissue into a 1.5 ml Eppendorf tube.

3. Add 500 µl of lysis Buffer for soft tissues (see Annexure I; 1.0 ml for every 500 mg of tissue) into the tube along with 20µl of 10 mg/ml Proteinase K solution (to a final concentration of 0.4 mg/ml) and SDS to make final concentration to 2% and mix gently. Incubate overnight at 56°C to ensure that the lysis is complete prior to the addition of phenol.
4. After digestion, briefly spin tubes and add one volume of phenol to the lysate and mix the contents by inverting the tube(s) for 10 minutes.
5. Centrifuge sample at 12,000 rpm for 10 minutes. Two phases can be observed after centrifugation: the upper aqueous phase and lower organic phase, with an interphase containing proteins.
6. Transfer the aqueous phase with the DNA to a clean tube taking care not to touch the interphase and the organic phase. Discard rest of the solution. Add an equal volume of 1:1 phenol/Chloroform : Isoamyl Alcohol mixture to the aqueous phase. Homogenize the mixture by inverting the tube for 10 minutes.
7. Centrifuge the sample again at 12,000 rpm for 10 minutes. The two phases will again form with a considerably finer interphase. Repeat steps (6) and (7) till the interphase disappears.
8. Transfer the aqueous phase to a new tube and mix with one volume of chloroform (chloroform removes phenol residues that may have remained in the sample). Mix the aqueous phase and chloroform by inversion for about 10 minutes.
9. Centrifuge at 12,000 rpm for 10 minutes. Precipitate the aqueous phase in a clean tube with 2 volumes of isopropanol. Incubation of the mixture for 5-10 minutes is optional, but may improve the precipitation of DNA.
10. Centrifuge at 12,000 rpm for 10 minutes to pellet the DNA. Discard the isopropanol. The DNA pellet should be visible at the bottom of the tube.
11. Wash the precipitate with 500µl of 70% ethanol. Centrifuge the sample at 12,000 rpm for 10-15 minutes. Optionally, a second wash can be done with ethanol to maximize sample purification.
12. Remove the ethanol and leave the DNA precipitate to dry. Finally, suspend the DNA in an appropriate volume of TE buffer or distilled water.

Blood and blood stains

1. 1-2 cm of bloodstain, 10 to 50µl whole blood, or 2 to 10µl buffy coat (layer of anticoagulated blood, containing WBCs and platelets) is required for this protocol.
2. Collect the blood stains in a sterile Petri dish and transfer into a sterile 1.5 ml tube.
3. Add appropriate volume of PBS to the stain and elute the stains for 2-4 hours at 56°C.
4. Spin the tube at 10,000 rpm for 10 minutes to collect the pellet.
5. Homogenize the pellet in BSS lysis buffer in a glass homogenizer and follow steps (4) to (12) in previous section.

Bone, Tusk, Teeth and Horns

1. One small piece of tooth or approximately 2 to 3 cm of bone/tusk/horn piece is suitable for DNA isolation. The exterior surface of each sample should be cleaned thoroughly of all debris. Bones may be cleaned on surface using sand paper to remove debris. Teeth may be cleaned with sterile distilled water, and bleach unless there are surface fractures, in which case only sterile water is used. Bone and teeth samples are prepared by crushing them into a fine powder. This can be achieved by using a drill machine with a stainless-steel chamber that is cleaned with bleach between each sample. Approximately 0.5g of sample should be placed into each 1.5 ml tube.
2. Incubate the bone and teeth scrapings in 0.5 M EDTA (pH 8.0) at room temperature for 48 hours.
3. After incubation, homogenize the bone scrapings and spin at 10,000 rpm for 15 minutes at 15^{o}C. Discard the supernatant and collect the pellet.
4. Wash the pellet thrice with 1X Phosphate Buffer Saline by repeated centrifugation and discard the supernatant (this ensures removal of EDTA).

   Add appropriate volume of Bone lysis buffer I, add proteinase-K and SDS to a final concentration of 2%, incubate at 37^{o} C for overnight, and follow steps (4) to (12).

Hair

1. Fill a clean 50ml beaker with autoclaved DW.
2. Pick up a single hair with a pair of forceps that has been cleaned previously with 80% alcohol.
3. Wash each hair to be analysed separately by immersing in fresh DW.
4. Use a clean scalpel and cut approximately 1cm portion from the root end of the hair.
5. Take the hair sample into an autoclaved 1.5ml tube. Add 0.5ml of digest buffer.
6. Add 20 µl of 1M DTT (to a final concentration of about 40 mM) and 15 µl of 10mg/ml Proteinase-K solution (to a final concentration of about 0.3 mg/ml). Vortex for 30 seconds.
7. Incubate at 56^{o}C for 6-8 hours or overnight until the hair is completely dissolved. Vortex for 30 seconds.
8. Spin the sample in a microcentrifuge for 1 minute at 12,000 rpm at room temperature to remove any pigment and particles.
9. Follow steps (4) to (12) in previous section.

Feathers

1. Fill a clean 50ml beaker with autoclaved DW.
2. Pick up a single feather with a pair of forceps that has been cleaned previously with 80% ethanol.
3. Wash each feather to be analysed separately by immersing in fresh DW.

4. Use a clean scalpel and cut the tip of the root end (calamus) of the feather.
5. Cut the tip in several small pieces and take into an autoclaved 1.5ml tube. Add 0.5ml of digest buffer.
6. Follow steps (4) to (12) in previous section.

QUANTIFICATION AND QUALITY CHECK OF DNA

UV absorbance (Spectrophotometer) method

At 260 nm, an absorbance of 1 measured in a cuvette with a 1-cm path length is indicative of double stranded DNA at a concentration of approximately 50 µg/ml. The ratio of absorbance at 260 nm to 280 nm is a useful indication of DNA purity. Values for DNA solutions of 1.8 to 1.9 and for RNA solutions of 1.9 to 2.0 are acceptable. The presence of protein, which absorbs at 280 nm, decreases the ratio as does the phenol, another likely contaminant.

Estimating DNA concentration using a spectrophotometer

1. Set blank using only 1 ml of TE buffer
2. Take 10 µl of DNA solution and dilute it by adding 990 µl of TE Buffer.
3. Mix well and measure absorbance values at 260 nm, for DNA purity take absorbance at 260 nm and 280 nm in a UV spectrophotometer and find the ratio A_{260}/ A_{280}.

Estimating DNA concentration using a NanoDrop spectrophotometer

DNA quantity and quality can be checked using a 0.8% agarose gel and an UV Nano-drop spectrophotometer. The Nano-drop ND 1000 (Fig 10) is a full spectrum (220-750 nm) spectrophotometer that measures the absorbance of as less as 1µl of sample with high accuracy and reproducibility. It utilizes a retention technology that employs surface tension alone to hold the samples in place.

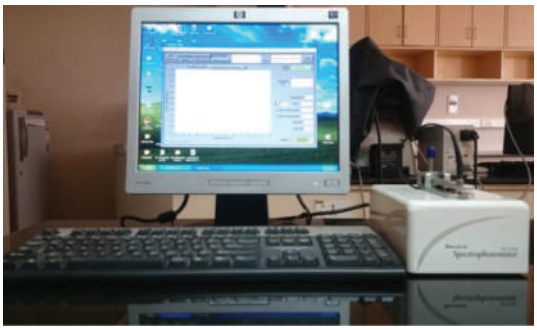


Figure 10. UV NanoDrop 1000 Spectrophotometer

Agarose Gel Electrophoresis

It is a method which is routinely used for separating proteins, DNA or RNA. Nucleic acid molecules are separated with the help of an electric field where negatively charged molecules migrate toward anode (positive electrode). DNA migration is determined majorly by its molecular weight where molecules having a lower molecular weight migrate faster than larger ones (Sambrook and Russell, 2001). To visualize nucleic acid molecules in agarose gels, intercalating dyes like ethidium bromide (EtBr) or SYBR green are commonly used which position themselves between the nitrogen bases of DNA and fluoresce under UV light.

Steps involved in agarose gel electrophoresis are as follows (Composition of reagents used is described in Annexure II):

1. Wash the appropriate sized gel tray and comb (Fig 11), seal the ends of the tray to prevent the gel from flowing out, place the comb and leave it on an even surface.
2. Weigh agarose powder and mix with electrophoresis buffer (1X TAE) to the desired concentration (0.8-1% for genomic DNA) and then boil it in a microwave oven.
3. Add EtBr to the solution (to a final concentration of 0.5µg/ml) and mix thoroughly to facilitate visualization of DNA under UV after electrophoresis.
4. After cooling the solution to about 60^{o}C, pour it into the casting tray with the comb and allowe to cool down to room temperature.
5. After the gel cools, keep it horizontally into the electrophoresis chamber and cover with sufficient 1X TAE buffer.
6. Remove the comb carefully so as not to damage the bottom of the wells.
7. Mix about 3-5 µl of DNA with the loading buffer (1 µl dye containing 1X Bromophenol blue) and load into the wells.
8. Connect the electrodes and switch on the DC power pack set at 50-70 V (Fig 12).
9. Negatively charged DNA migrates towards the anode (positive electrode). The distance migrated in the gel can be judged visually by monitoring the migration of the tracking dye.
10. Load λ phage DNA of known concentration (50ng/µl) along with the samples to compare and visually quantify the DNA. Visualize under UV light in a transilluminator and take pictures of the gel in a gel documentation system.
11. Visualization of DNA: Electrophorese the DNA samples till the tracking dye reaches the middle of the gel (Figure 13), and observe the gel under UV and document.

Figure 11.
An electrophoresis tray with combs

Figure 12.
Electrophoresis unit along with power pack

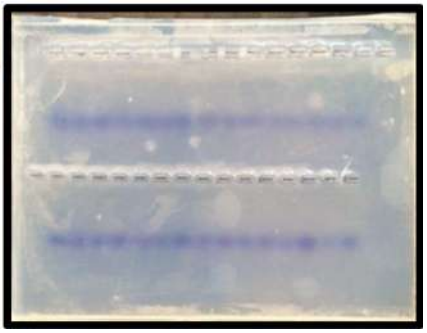


Figure 13.
An electrophoresis tray with agarose gel

PRECAUTIONS FOR DNA HANDLING

Storage of DNA

DNA can be stored in a concentrated form at 4°C for quite a long time (about 12 months) without affecting its quality. But for long storage periods, it is recommended to store DNA at - 20°C. DNA in diluted form should be strictly stored at 4°C, as frequent thawing and freezing leads to physical degradation of DNA and drastic reduction in quality. A working DNA solution preferably should be in 0.1X TE, wherein EDTA concentration is 0.1 mM, which avoids inhibition of subsequent enzyme activity.

Transport of DNA

DNA can be transferred from one place to another at room temperature in air-dried or vacuum-dried form without any damage to quality and quantity, if properly sealed and protected from moisture. DNA dissolved in TE also can be transferred over short distances in ice. But for long distance transport of DNA at room temperature, it is recommended to re-precipitate using absolute alcohol containing Sodium acetate (pH 5.2) in a ratio of 50:1 and transfer in 80% ethanol as pellet.

Precautions for working with biological specimens

1. Wear gloves whenever handling biological specimen.
2. To avoid generation of aerosols, we recommend that steps involving mixing of blood or cells derived from blood be performed in a biological safety cabinet.
3. Decontaminate work surface daily; contaminated items should be autoclaved before disposal.
4. Phenol is extremely corrosive to skin and eyes and can cause severe burns. Wear safety glasses and chemical-resistant gloves when working with phenol. Procedures involving phenol should be performed in a chemical fume hood and the subsequent waste stored in the hood.
5. Chloroform is a carcinogen and is toxic by inhalation, skin absorption and ingestion. Procedures involving chloroform should be performed in a chemical fume hood and the subsequent waste stored in the hood.

2

SPECIES IDENTIFICATION

Background

Mitochondrial DNA (mtDNA), a very small fraction of our genome, is uniparentally inherited. It is a double stranded circular molecule (Fig 14) with a length of nearly 15-17 kilo base pairs. Special characteristics of mtDNA include high copy number, lack of recombination, maternal inheritance and a higher evolutionary rate. The mutation rate in the non-coding regions is higher than that of coding regions (Pakendorf & Stoneking 2005; Howell et al. 2007) like any other part of the genome. Several segments of mtDNA are conserved (Boonseub et al. 2009). Variations in the mtDNA are a result of sequential accumulation of mutations through evolution. Variations within and between species can be assessed by sequencing partial or complete mtDNA to establish the identity for various evolutionary lineages of different species.

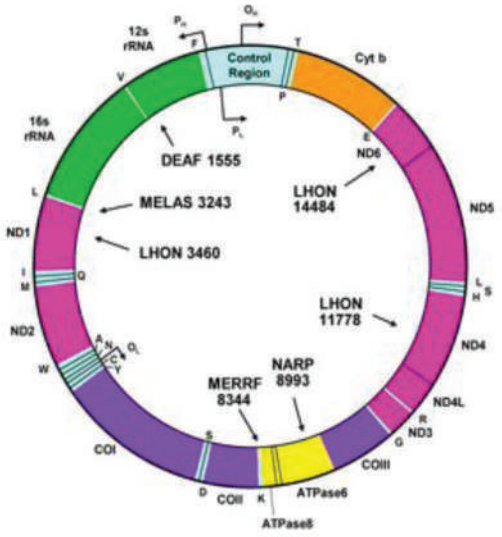


Figure 14.Schematic representation of the mtDNA. Protein-coding genes present in the mtDNA are: Cytb- cytochrome b; COI, COII and COIII- Subunits I, II and III of the cytochrome oxidase; ND1-6-subunits 1 to 6 of the NADH reductases; tRNA are represented by their three letter amino acid abbreviations (Source: https://ghr.nlm.nih.gov/mitochondrial-dna#ideogram).

Purpose

The purpose of this chapter is to explain the working of a universal genetic marker for species identification by analyzing different biological material (i.e. the DNA isolated from confiscated animal remain of unknown origin) using our in-house database and public databases such as GenBank in NCBI.

Scope

This method is suitable for the identification of DNA from various biological materials of unknown origin. It has been tested against a broad taxonomic range of animal species (Verma & Singh 2003). The method is designed to work with samples like skin, meat, blood, bones, feathers etc. It is also successful with cooked meat products, but success is dependent on the intensity of cooking.

Principle of the Method

Mitochondrial cytochrome b (Cytb) is a highly-conserved region and is effective in identifying many species groups (Parson *et al.* 2000; Hsieh *et al.* 2001; Branicki *et al.* 2003). The region of the mcb that is used for universal identification of animal species consists of 472 base-pairs (Verma & Singh 2003).

Chemicals and Equipment

The sections below report all the reagents and equipment required in mitochondrial DNA analysis for species identification.

Plastic-ware: It is essential that all plastic-ware is sterile before use. (Table 2)

Table 2. List of plastic ware required for PCR

Item	Volume
PCR tubes	0.2ml
Eppendorf tubes	1.5ml
Pipette tips (filtered)	2, 200 & 1000µl

Instruments: The following items of equipment are required to undertake the analysis. (Table 3)

Software:CodonCode Aligner (www.codoncode.com) for sequence alignment and editing (Internet access is required to utilize the NCBI database https://www.ncbi.nlm.nih.gov/nucleotide/)

Primers: Forward - mcb398, Reverse - mcb869: these mitochondrial markers being used for species identification were developed by Verma and Singh (2003); the numbers 398 and 869 refer to the positions of 5′ base of the primers in the complete cytochrome b sequence of Antelope cervicapra, NCBI Accession no. AF022058).

Polymerase chain Reaction

Prepare the PCR reaction mix as given in Table 4.

1. Alongside every set of reactions ensure a negative control (i.e. DW) and a positive control (i.e. a DNA sample from a known species).
2. Place the tubes in the PCR machine and run the PCR programme specifying conditions mentioned in Table 5.
3. Once completed the PCR products can be stored at 4°C. But for long term storage (i.e. more than a week) freezing at -20°C is recommended.

Precaution: It is essential to use pipette tips that are sterile and fitted with filters.

Table 3. List of equipment required for PCR

Item	Suggestion of make
Vortex	Labnet VX100
Thermo cycler	Applied Biosystems 2720, Biorad DNA Engine, Eppendorf AG
Automated DNA Sequencer	3730 DNA Analyzer
DNA quantifier	Nanodrop ND-1000
Gel documentation Systems	Biorad
Micro centrifuge	Eppendorf mini spin plus
Electrophoresis apparatus	Amersham Pharmacia Biotech

Table 4. Composition of reagents in PCR for amplifying cytb sequence

Component	Volume (in µl)
10x PCR buffer	1.3
25mM MgCl2	1.0
10x BSA	1.1
10mM dNTPs	1.2
mcb398F (5 pmoles/ µl)	0.5
mcb869R (5 pmoles/ µl)	0.5
Taq polymerase (3 U/ µl)	0.15
DW	6.25
DNA(25 ng/µl)	3.0

Table 5. Thermal conditions for PCR reaction

Steps	Temperature	Duration	
Initial denaturation	95°C	5 min	
Denaturation	94°C	45 s	**35 cycles**
Annealing	52°C	50 s	
Extension	72°C	1 min 20 s	
Final extension	72°C	7 min	
Hold	15 °C	10 min	

Table 6: Sequencing PCR conditions

Temp	Duration
96°C	10 seconds
50°C	5 seconds
60°C	4 min
4°C	10min

Analysis of PCR products by Agarose gel electrophoresis

Agarose gel electrophoresis of DNA is a standard technique in molecular biology for quantitative and qualitative analysis of DNA.

1. Make 2% agarose gel (Annexure II).
2. Once set, load 3µl of the PCR product into the well mixed with loading dye.
3. Include appropriate size standard in one lane (100-bp ladder).
4. Run at 100 V for approximately 15-20 minutes (depending on size of gel), ensuring the DNA does not run off the gel.

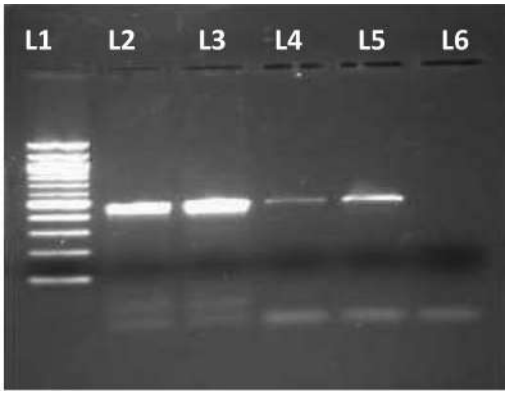


Figure 15. Gel picture showing PCR (472 bp) products. L1-100 bp ladder, L2-L4 Samples, L5- Positive control, L6-Negative control indicating a successful amplification

5. Visualize the amplified fragments in a UV transilluminator (with appropriate safety precautions); if the PCR reaction was successful, the positive control will have a single bright band of approximately 472 bp in length (Fig 15). The approximate length of the amplified DNA is estimated by taking a 100 bp ladder as the reference. The ladder comprises of fragments of known length. The length (amplicon size) of PCR product is identified by matching the size of the fragment in the ladder, that has migrated to the same extent. The negative controls should not display amplifications. A band of the desired fragment size (472 bp) in the lanes corresponding to your samples indicates successful amplification.
6. Keep a permanent record of your gel (electronic and/or hard copy) as proof that the PCR amplification was successful and contaminant free.

DNA SEQUENCING

Before sequencing the PCR products, estimate the concentration of your PCR product. This can be done from the record you made of your PCR products when run on the agarose gel, by comparing the brightness of the bands to the size standard that was run (provided it has a standard concentration of DNA). Each PCR product should have two sequencing reactions; one with the forward primer and a second utilizing the reverse primer. Ensure that the PCR products are purified prior to the sequencing reaction. This can usually be done by ExoSAP Treatment (USB® ExoSAP- IT® PCR Product cleanup).

PCR cleanup protocol (for 96-well plate)

1. Remove ExoSAP-IT® reagent from -20°C freezer and keep on ice throughout the procedure.
2. Add equal volumes of PCR product and ExoSAP master mix (Mix 50µl of ExoSAP with 150µl of DW)in each well followed by short spin.

3. Incubate at 37 °C for 15 minutes to degrade left-over primers and nucleotides followed by incubation at 80 °C for 15 minutes to inactivate ExoSAP-IT® reagent.
4. The PCR product is now ready for Sequencing PCR.

Sequencing PCR protocol

1. Prepare a Big Dye/Big Dye buffer premix: 25 µl of Big Dye with 175µl of DW in a 1.5ml Eppendorf tube. Remember to prepare the mixture for an extra reaction. For instance, if you have 5 samples, prepare the reaction mixture for 6 samples to accommodate for pipetting error.
2. Dilute the 100 pM stock solution of sequencing primer 1:10 with sterile DW. Pipette 2µl of the diluted sequencing primer into PCR tubes/plate.
3. Pipette 1.8µl of the Big Dye Ready Reaction Termination Mix into each tube.
4. Place the tubes in the thermal cycler. Program the thermal cycler as presented in Table 6.

Precipitation and post-processing of DNA for sequencing

1. Precipitation mix: 3ml 100% ethanol with 250µl of sodium acetate.
2. Add 25µl of this mix into each well, mix by vortexing briefly, leave at room temperature for 30 minutes.
3. Spin tubes for a minimum of 20 min at 4000 rpm in a micro centrifuge.
4. Aspirate the supernatants completely with a separate pipette tip for each sample or gently invert the plate on a tissue paper.
5. Add 100 ml of 70% ethanol to the tubes and vortex briefly, centrifuge as before for 20 min at 3500 rpm, and aspirate the supernatants as in the previous step.
6. Spin the plate inverted, at 200 rpm for 30 seconds. Air-dry the plates on the work bench for an hour. Plates, covered with tissues, can also be left to dry overnight.
7. Once the plate appears dry, suspend the wells with 25µl of Hi-Di formamide™ (can also use 12.5µl of formamide and 12.5µl of DW).
8. Now place the plate in Automated DNA sequencer.

Automated DNA Sequencer (for example ABI 3730) is a 96-well capillary electrophoresis chamber designed to detect and sequence amplified DNA and convert data into an interpretable, graphical display called an electropherogram. A modified PCR reaction called the sequencing PCR is conducted for the products (one well for one product) by using a single primer as opposed to two primers added during normal PCR. Therefore, sequencing PCR amplifies a single strand of the double-stranded DNA of the PCR amplicon. The single strand of DNA is then electrophoresed in the Automated DNA sequencer. ddNTPs are labeled with fluorescent

probes to enable detection by a fluorescent probe detector inside the sequencer and converted to an electrical signal by a CCD camera. Data corresponding to the amplified DNA fragments is ultimately represented by peaks of the electropherogram.

Raw data processing

The ABI files can be viewed and edited with CodonCode Aligner™.

1. Open the CodonCode software by clicking on the CodonCode.exe icon
2. Open the ABI (. abi) file of your sample by selecting the file menu and the open option followed by creating new project option. Select the ABI sample sequences from the folder containing sample sequences.
3. This will open a window within the software; the electropherogram, i.e. the sequence traces or peaks (Fig 16) corresponding to the signal from each of the nucleotides in the DNA sequence.
4. To view the trace within the software, both zoom and relative peak height function is present that can be adjusted to your preference.
5. The sequence can be visually checked to ensure that the trace is of high quality and the sequence reaction has worked successfully (Fig 17). In case of reaction failure or contamination, the peaks will look weak and/or it will be impossible to clearly resolve a single peak at each nucleotide position.
6. Often the quality of the sequencing reaction is poor at either ends of the trace (Fig 18). In this case, the ambiguous region at either end can simply be deleted, just leaving the high-quality sequence (i.e. delete the flanking sequence at each end until you are confident that you can easily call each peak).
7. Ambiguous nucleotides may also arise within the sequences (Fig 19). Do not delete them. Edit the corresponding nucleotide with respect to the compliment strand sequence.
8. Generating Consensus Sequences - Each of the samples should be sequenced in both the forward and reverse directions, meaning these complementary/overlapping sequences can be combined to generate a consensus sequence. This serves as an important way of checking the accuracy of the sequence, and can help to remove any ambiguous bases and generate a longer total sequence.
9. It is necessary to save the edited sequences. To save the contigs generated, click on the nucleotide sequence window (so it is selected as the active window) and save the sequence. This is done in the file menu, select the option “save project as” in the toolbar by selecting “Save As” (Fig 20). The file can be renamed (e.g. with the name of the original sample with indication as to whether the sequence was generated with the forward or reverse primer).
10. The project file must be saved and exported in FASTA format (Fig 21). To export the consensus sequences of contigs, select contigs of interest in the project view, and then choose “Export Consensus Sequences” from the “File” menu.

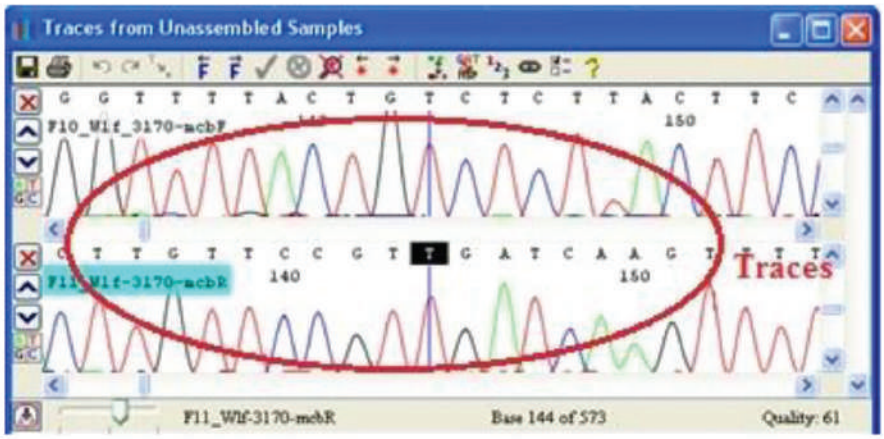


Figure 16. Sequence showing peaks/ traces

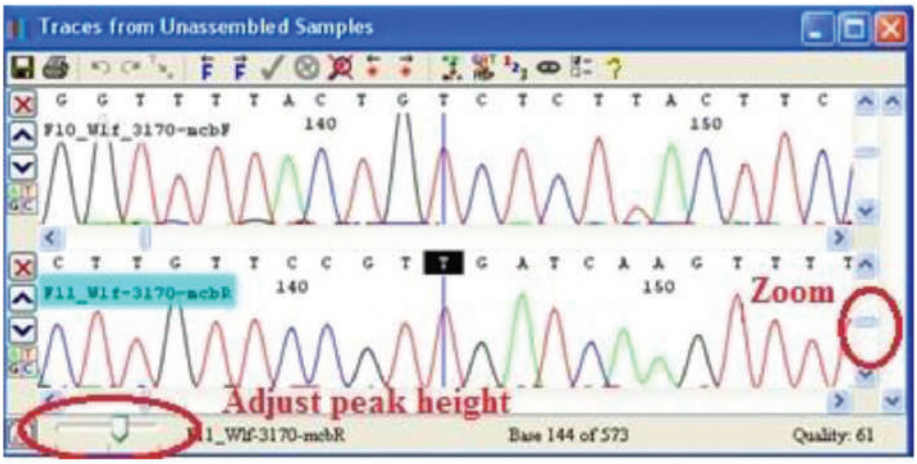


Figure 17. Trace view: Edit and view traces

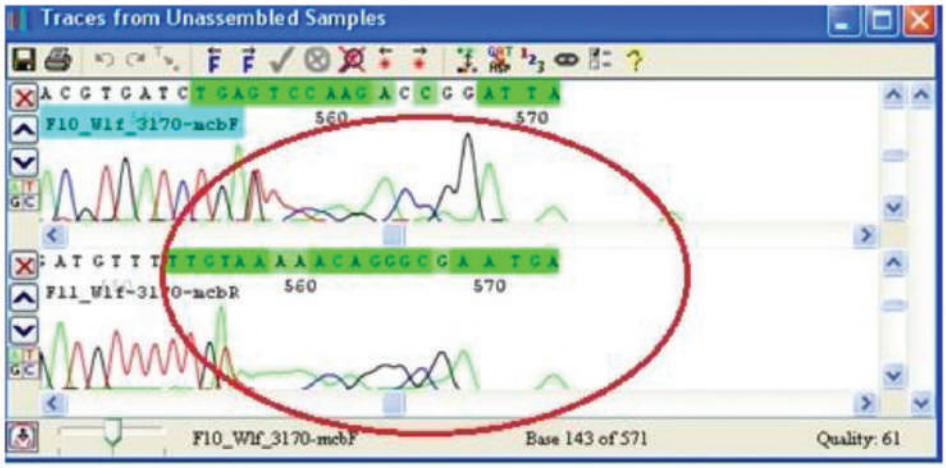


Figure 18. Ambiguous traces in the flanking region

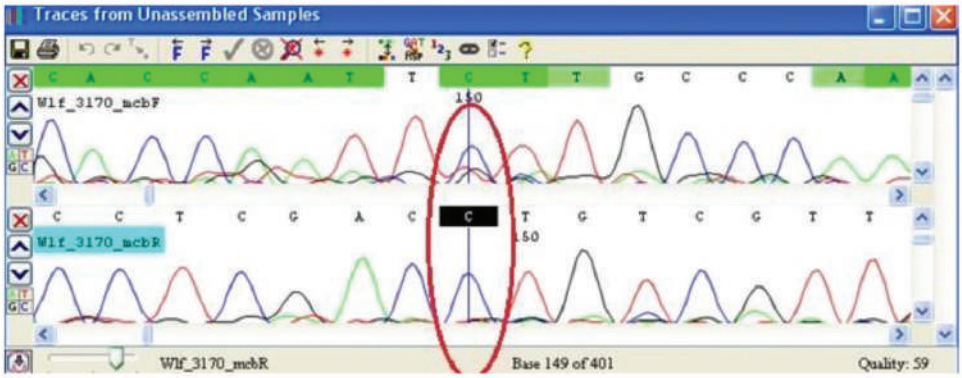


Figure 19. Data showing ambiguous traces in the middle of the sequence

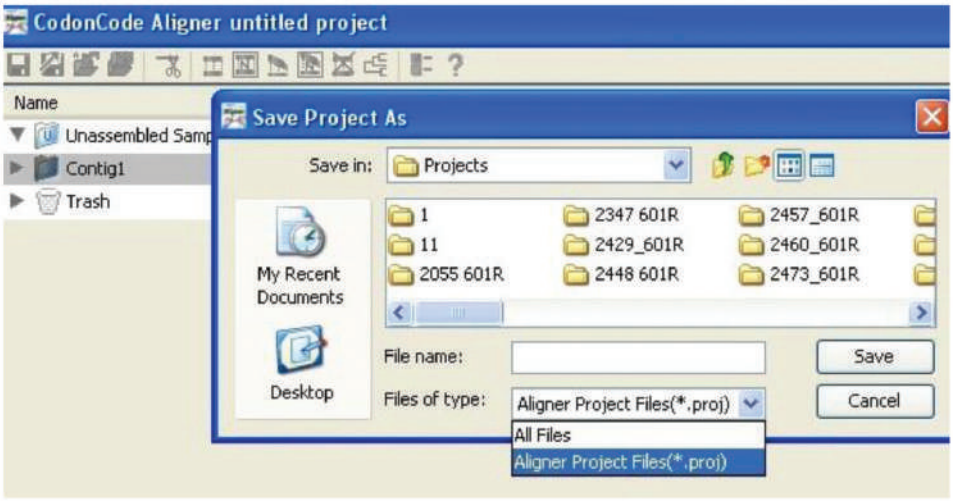


Figure 20. Project file save option window

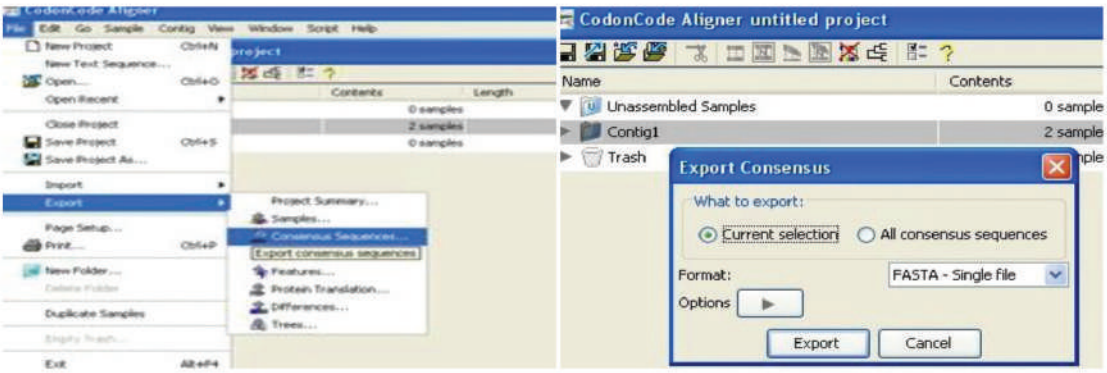


Figure 21. Exporting Assemblies window

Species Identification using BLAST algorithm of NCBI database

To identify which species your consensus represents, the mcb sequence generated is compared to the free access data in the NCBI project using BLAST tool (Fig 22).

This page acts as a portal which allows the consensus sequence generated in the laboratory to be referenced against the entire NCBI database. Various search options are possible that relate to different collections of reference data, but the default settings provide an excellent initial step at identifying the species (Fig 23).

The browser will update and give you the results of the search, revealing the records contained in the database that yields the closest match in terms of sequence similarity (Fig 24). First, it is important to save a screen grab of the results as proof of the result, something like the picture below.

When you scroll down, this screen also contains information that will allow a confident identification to be made from your sequence (Fig 25). The right corner shows the similarity scores of the top 99 matches that show the percent similarity for each of 99 top matching records in the database against your consensus sequence. The example below clearly illustrates that there is a 100% sequence similarity between our example consensus sequence and the *Panthera leo persica* records.

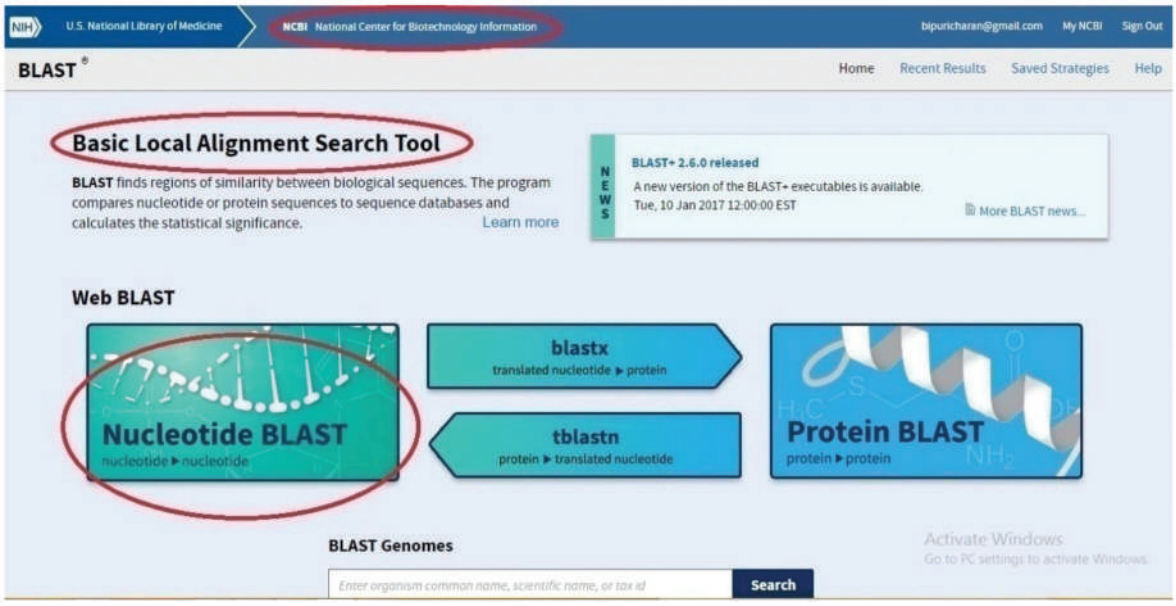


Figure 22. NCBI BLAST home page

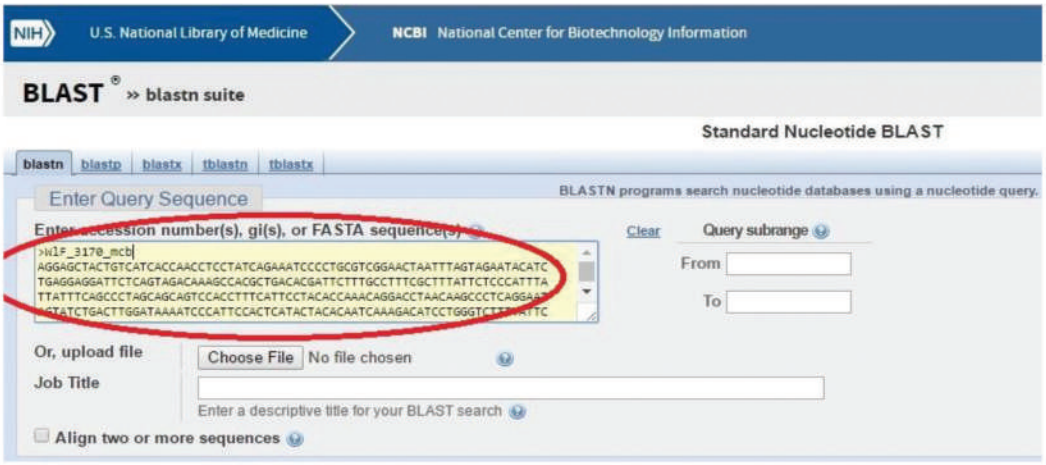


Figure 23. Nucleotide query window

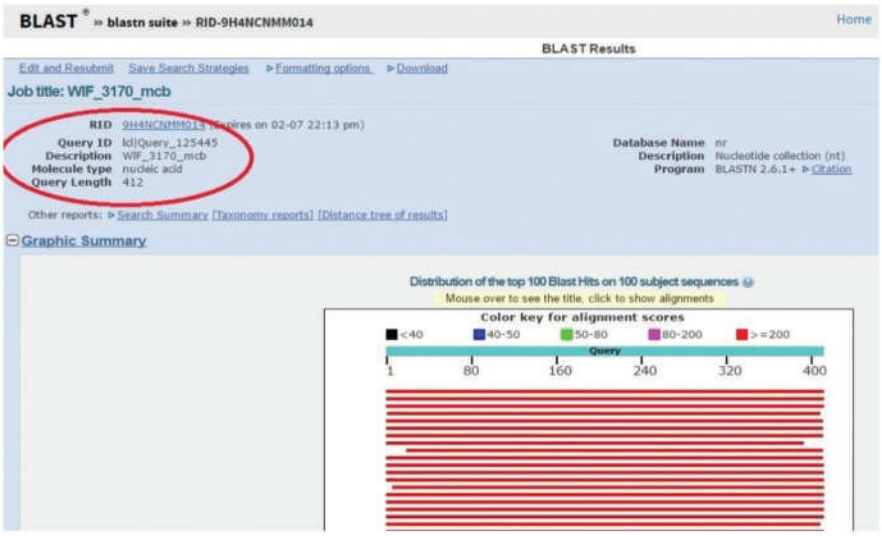


Figure 24. Result window

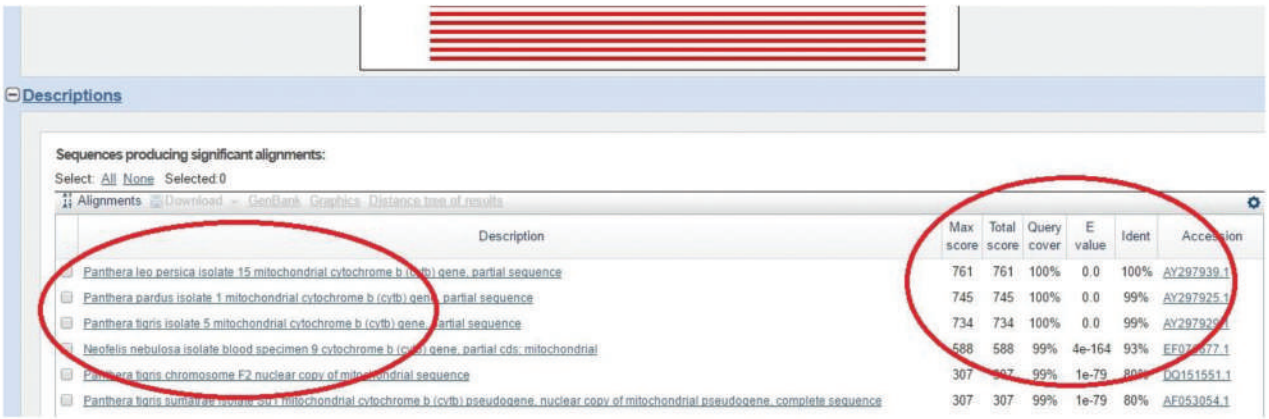


Figure 25. Similarity scores: Query Sequence aligned with the best hit from the database

Species-specific Identification

PCR assay with tiger-specific cytochrome b primers (TIF and TIR) developed to establish tiger species identity and differentiate tiger samples from those of sympatric carnivores, like leopards, can be used to identify tiger samples (Bhagavatula & Singh 2006).

Primer name	Sequence	Annealing Temperature	Product size (bp)
TIF	5′-ATAAAAAATCAGGAATGGTG-3′	59°C	162
TIR	5′-TGGCGGGGATGTAGTTATCA-3′		

PCR amplifications with tiger-specific cytochrome b primers TIF/TIR can be carried out in 15µl reactions with the following final composition:

PCR Component	Volume (in µl)
10X Ex Taq buffer (TaKaRa™)	1.5
2.5mM dNTPs (TaKaRa™)	1.5
10X BSA (New England Biolabs™)	1.5
5 pm/µl TIF primer	0.7
5 pm/µl TIR primer	0.7
Taq polymerase (TaKaRa Ex Taq HotStart™)	0.15
Template DNA (20 to 50 ng/µl)	1
DW	7.95
Total	**15**

Carry out PCR reactions with the following conditions:

Steps	Temp	Duration
nitial Denaturation	95°C	2 min
Denaturation	94°C	30 s } **45 cycles**
Annealing	59°C	30 s
Extension	72°C	30 s
Final Extension	72°C	10 min
Hold	15°C	-

PCR products can be visualized using agarose gel electrophoresis (2.5% agarose gel) and only the tiger positive samples may be subjected to further analysis.

Evaluation of DNA yield from field samples using qPCR

Since faecal samples yield unpredictable amounts of low quality DNA which can lead to subsequent genotyping errors, quantifying the amount of DNA in each tiger- positive sample by qPCR using primer set CmycEx3-71F and CmycEx3-223R(which amplifies 191bp of exon 3 of *c-myc* proto-oncogene in felids) is advisable.

Primer name	Sequence	Annealing Temperature	Product size (bp)
CmycEx3- 71F	5′-CCTTAAGAGATGCCACGTGC-3′	60°C	191
CmycEx3- 223R	5′-TGTGCGTCCGCCTCTTGTCG-3′		

- Perform amplifications in triplicates with 8µl reaction mixture containing the following final composition:

PCR Component	Volume (in µl)
SYBR Green (Invitrogen)	4
forward primer (5pM/ µl)	1
reverse primer (5pM/ µl)	1
DNA extract	2
Total	8

- Set up qPCR in a Real Time PCR System with the following PCR conditions:

Steps	Temp	Duration	
Initial Incubation	50°C	2 min	
Initial Denaturation	95°C	10 min	
Denaturation	95°C	15 s	*
Annealing	60°C	30 s	
Extension	72°C	30 s	
Denaturation	95°C	15 s	**
Annealing	60°C	15 s	
Extension	95°C	15 s	
Hold	15°C	-	

*40 cycles

**Final dissociation cycles/Melting curve

- DNA for the standard curve should consist of 10 dilutions (20ng, 4ng, 800pg, 160pg, 32pg, 6.4pg, 1.28pg, 0.25pg, 0.05pg, 0.01pg and 0.002pg per µl) of good quality DNA. Quantify 20 ng/µl of DNA initially by nanodrop and then serially dilute the DNA.
- Include a negative control with each standard curve, and three such controls in each plate of sample extracts.
- Reject the Standard curve, if correlation coefficient of the trendline is < 0.95.
- Calculate the DNA concentrations in the extracts from slope and Y-intercept (Yint) of the trendline obtained from standard curve, plotted as log of DNA concentrations versus Ct values: **DNA concentrations = 10(*Ct-Yint*)/slope).**
- Grade the Samples based on number of successful qPCRs.

Grade	DNA concentration in pg/µl	
	qPCR	Nanodrop reading
I	>20	200 – 45,000
II	1-20	300 – 37,400
III	<1	100 – 43,000
IV	Undetectable	150 – 35,000

3

MICROSATELLITE GENOTYPING FOR INDIVIDUAL IDENTIFICATION IN TIGER POACHING CASES

Samples which yield sufficient quantities (>5 pg/µl) of usable DNA (Morin et al. 2001) may be genotyped at (thirteen microsatellite loci) - ten tetranucleotide (F37, F42, F53, F115, F124, F141, F146, Fca391, Fca424, Fca44); one trinucleotide (E6) and two dinucleotide microsatellite loci (Fca96, E7).

Locus	Primer Sequence	Repeat type	Annealing Temperature	Size Range (bp)
F37	5′-CGCCTTTCTCACATTACCAT-3′	Tetra	58°C	225–240
	5′-CACTGACAGATCTGATCCTG-3′			
F42	5′-CCCACGTGGACTAATCAAAT-3′	Tetra	62°C	200–240
	5′-CACTGCACAAATTAAGAGGC-3′			
F53	5′-GTTGGGAGTAGAGATCACCT-3′	Tetra	58°C	165–190
	5′-GAAAAAGACTCCTGCTTGCA-3′			
F115	5′-CTCACACAAGTAACTCTTTG-3′	Tetra	54°C	175–195
	5′-CCTTCCAGATTAAGATGAGA-3′			
F124	5′-TGCTGGGTATGAAGCCTACT-3′	Tetra	52°C	200–220
	5′-ATTGCCTCAACTACCTAGGC-3′			
F141	5′-CATTCTGCTCTCAGAGCATG-3′	Tetra	52°C	255–285
	5′-GTCTGGGTCTTGTAACTCCT-3′			
F146	5′-TTACGGTCTCTCCACAAGTC-3′	Tetra	50°C	140–160
	5′-GAACCAGGTGATGAGAACTG-3′			
Fca391	5′-GCCTTCTAACTTCCTTGCAGA-3′	Tetra	50°C	195–220
	5′-TTTAGGTAGCCCATTTTCATCA-3′			
Fca424	5′-TGGAAAAATGTGGAATACTGAA-3′	Tetra	54°C	150–175
	5′-CCAATTTGTAGTGACATCCCC-3′			
Fca441	5′-ATCGGTAGGTAGGTAGATATAG-3′	Tetra	52°C	145–160
	5′-GCTTGCTTCAAATTTTCAC-3′			
Fca96	5′-CACGCCAAACTCTATGCTGA-3′	Di	52°C	195–205
	5′-CAATGTGCCGTCCAAGAAC-3′			
E6	5′-CCTGGGGATAATAAAACTAGTA-3′	Tri	52°C	135–160
	5′-CATGAATGAATCTTTACACTGA-3′			
E7	5′-GCCCCAAAGCCCTAAAATAA-3′	Di	51°C	135–150
	5′-GCATGTCGGACAGTAAAGCA-3′			

- Amplify all microsatellite loci in triplicates in 15µl reaction volumes consisting of:

PCR Component	Volume (in µl)
10X Ex Taq buffer (TaKaRa™)	1.5
2.5mM dNTPs (TaKaRa™)	1.5
10X BSA (New England Biolabs™)	1.5
5 pm/µl Forward primer (Fluorescently labeled)	0.7l
5 pm/µl Reverse primer	0.7
Taq (TaKaRa Ex Taq HotStart™)	0.15
Template DNA (20 to 50 ng/µl)	1
DW	7.95
Total	**15**

- Carry out PCR reactions with the following conditions:

Steps	Temp	Duration
Initial Denaturation	95°C	2 min
Denaturation	94°C	20 s
Annealing	T_a	30 s } **45 cycles**
Extension	72°C	30 s
Final Extension	72°C	10 min
Hold	15°C	-

- Samples with ambiguous results or with poor amplification success must be further amplified three times.
- Perform All PCR steps, except addition of template DNA, in a hood that is UV-irradiated before and after use to avoid contamination.
- All PCR reactions including positive and negative controls and PCR products may be electrophoresed on Genetic Analyser and size the alleles relative to an internal control (500 LIZTM, Applied Biosystems) using GeneMapper software version 3.7 (Applied Biosystems). Only samples which amplify at a minimum of eight loci may be included in the final data set.
- Analyse all allelic data in Microsoft EXCEL spreadsheets. Carry out allele frequency analysis, estimates of probability of identity (PID) and PID (sib) using CERVUS version 3.0. Identify unique genotypes by the Identity Test in CERVUS. Pool samples which match at a minimum of eight loci to create consensus genotypes.
- Re-examine samples which have mismatches at up to three loci by PCR for possible genotyping errors.
- Also repeat PCR in triplicates at the unamplified loci to obtain complete genotypes. In case of heterozygous genotypes, each allele may be observed in a minimum of two independent PCRs, whereas in homozygous genotypes, each allele may be observed in four independent PCRs.

SEX IDENTIFICATION

Tiger positive samples which amplify at a minimum of eight microsatellite loci may be further subjected to PCR assays for sex identification with primer pairs targeting the zinc finger (ZuF) and amelogenin loci.

Primer	Sequence	Annealing Temperature	Product Size (bp)
ZnF	F: 5′-AAGTTTACACAACCACCTGG -3′	62°C	Male: 163, 166
	R: 5′- CACAGAATTTACACTTGTGCA -3′		Female: 166
Aml	F: 5′-CGAGGTAATTTTTCTGTTTACT -3′	52°C	Male: 194, 214
	R: 5′-GAAACTGAGTCAGAGAGGC -3′		Female: 214

- PCR amplifications with zinc finger and amelogenin primers can be carried out in 15 µl reactions with the following final composition:

PCR Component	Volume (in µl)
10X Ex Taq buffer (TaKaRa™)	1.5
2.5mM dNTPs (TaKaRa™)	1.5
10X BSA (New England Biolabs™)	1.5
5 pm/µl Forward primer*	0.7
5 pm/µl Reverse primer	0.7
Taq (TaKaRa Ex Taq HotStart™)	0.15
Template DNA (20 to 50 ng/µl)	1
DW	7.95
Total	**15**

*Forward primer for amplifying ZnF region is fluorescently labeled

- PCR reactions can be carried out with the following conditions :

Steps	Temp	Duration
Initial Denaturation	95°C	2 min
Denaturation	94°C	20 s
Annealing	T_a*	30 s } **45 cycles**
Extension	72°C	30 s
Final Extension	72°C	10 min
Hold	15°C	-

T_a* - 52°C for Aml markers and 62°C for ZnF markers

- Electrophorese PCR products obtained with amelogenin markers on 3% agarose gel (Male shows two bands at 194 and 214bp; Female shows single band at 214bp).
- Electrophorese PCR products obtained with zinc finger markers on an ABI 3730 Genetic Analyzer and size the alleles relative to 500 LIZ™ (Applied Biosystems) using GeneMapper software version 3.7 (Applied Biosystems) (Male shows peaks at 163 and 166bp; Female shows single peak at 166bp).

RELATEDNESS AND PATERNITY ANALYSES

- Estimate relatedness coefficient (R) for all pairs of individuals in a population with Queller and Goodnight estimator (Queller & Goodnight 1989) implemented in GENALEX 6.501 (Peakall & Smouse 2006).
- Values of R range between -1 and +1, and are indicative of the proportion of shared alleles which are identical by descent between pairs of individuals. Generally unrelated individuals have R values between -1 and 0.125, half-siblings have values between 0.125 and 0.375, and full siblings, or parent-offspring pairs have values between 0.375 and 0.625 (Queller & Goodnight 1989).
- ML-RELATE can also be used to calculate maximum likelihood estimates of relatedness (r) and to establish relationships from co-dominant genetic data (Kalinowski et al. 2006).
- Maximum likelihood estimates of relatedness are usually more accurate than other estimators and are useful to discriminate between four common pedigree relationships: unrelated, half-siblings, full-siblings and parent-offspring.
- Likelihood ratio approach in CERVUS 3.0.7 (Marshall et al. 1998) may be used to assess candidate mothers and fathers. Genotypes of suspected parent and offspring may be matched manually prior to maternity/ paternity analysis in CERVUS.
- The most likely parent for cub may be determined and statistical support of the results may be estimated assuming 90% sampling of candidate mothers/ fathers per cub and 1% genotyping error.

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ANNEXURE I

Preparation of reagents in Molecular Biology Laboratory

1) 1M Tris :Dissolve 121.1 g of Tris-HCl in 800 ml of H2O. Adjust the pH to the desired value by adding concentrated HCl.

Desired pH	Amount of conc. HCl
7.4	70 ml
7.6	60 ml
8.0	42 m

Check pH using a Tris compatible electrode or litmus paper and adjust. Bring volume to 1 liter. Dispense in 100ml aliquots and autoclave for 15min at 15 psi.

2) **5 M NaCl :** Dissolve 292 g of NaCl in 800 ml of H2O. Adjust volume to 1 liter with H2O, Dispense into 100 ml aliquots and autoclave for 15 min at 15 psi.

3) **10 M NaOH :** Add 40 g of NaOH slowly to 80 ml of H2O. Stir until completely dissolved. Bring volume to 100 ml and store in a plastic bottle. There is no need to autoclave.
Note: This is an exothermic reaction; the beaker will become quite hot and can break. **Use plastic beakers as a precaution.**

4) **Proteinase K (20 mg/ml):** 20 mg of proteinase K was dissolved in 1ml of autoclaved MilliQ water.

5) **20% SDS :** Dissolve 100 gm of SDS in 400 ml of autoclaved distilled water, heat gently and stir to aid dissolution and make final volume up to 500 ml. Dispense into 100 ml aliquots and store at RT. Do not autoclave.

6) **1M DTT :** Dissolve 15.45 g of DTT in 100 ml autoclaved distilled water and store at -20°C.

7) TE Buffer

Reagent	Volume
1M Tris-HCl	1.0 ml (10.0 mM)
0.5M EDTA	0.2 ml (1mM)

Make up to 100 ml with DDW.

8) **0.5M EDTA (pH 8.0) :**Dissolve 186.1g Na2EDTA.2H2O in 700 ml H2O. Adjust pH to 8.0 with 10M NaOH (~50 ml) and finally add DW to make up the volume to 1 liter.

9) **Ethidium Bromide (10 mg/ml):** Dissolve 10 mg of Ethidium bromide in 1ml of DW. Mix well and store it in dark bottles.
Precaution: Ethidium bromide is a carcinogen and must be handled carefully.

10) **3M Sodium acetate :** Dissolve 204 g of sodium acetate using a magnetic stirrer in 400 ml of DW, pH was adjusted to 5.5 with dilute acetic acid; volume made up to 500 ml and autoclaved.

11) **PBS (phosphate-buffered saline) 10x stock solution, 1 litre**

Reagent	Amount
Sodium chloride (NaCl)	80 g
Sodium phosphate (Na2HPO4.7H2O)	11.5 g
Potassium phosphate (KH2PO4)	2 g
Potassium chloride	20 g

Dissolve all the components in 2 litre bottle using magnetic stirrer in 500 ml of double distilled water and adjust the pH 7.4 with HCl; and make up final volume to 1000 ml and autoclave.

12) **50X TAE Buffer**

Reagent	Amount
Tris base	121g
0.5 M EDTA (pH 8.0)	50 ml
Glacial acetic acid	28.55 ml

Mix and make up the volume to 500 ml.

13) **6X loading dye :** 10% sucrose solution was prepared in autoclaved DW and 62.5 mg of Bromophenol blue was dissolved in it to make a stock of 25 ml.

Lysis buffers used in DNA isolation:

Reagent A

Reagents	Amount
Sucrose	109.54 g (320 mM)
1 M $MgCl_2$	5 ml (5 mM)
Triton X	10 ml (1%)
1 M Tris-HCl (pH-8)	10 ml (10 mM)

Make up to 1000 ml with DW.

Reagent B

Reagents	Volume
1M Tris-HCl (pH-8)	40 ml (400 mM)
0.5M Na-EDTA	12 ml (60 mM)
1M NaCl	15 ml (150 mM)

Make up to 95 ml with DW, autoclave. Then add 5 ml 20% SDS (1%)

Reagent C

Add 100 g of NaClO4 to 142 ml of DW to prepare 5M NaClO4. Do not autoclave.

Lysis buffer for stored blood:

Lysis buffer I

- 30 mM Tris-HCl (pH 8.0)
- 5 mM EDTA
- 50 mM NaCl

Lysis buffer II

- 75 mM NaCl
- 2 mM EDTA (pH 8.0)

Lysis buffer – BSS for blood stains and Swabs:

- 0.32 mM Sucrose
- 10 mM MgCl2
- 10 mM Tris-HCl
- 50 mM EDTA
- 100 mM NaCl

Lysis buffer for soft tissues

- 50 mM Tris-HCl (pH 8.0)
- 10 mM EDTA (pH 8.0)
- 100 mM NaCl

Lysis buffer for bone marrow:

- 100 mM Tris-HCl (pH 8.0)
- 10 mM EDTA
- 100 mM NaCl

Lysis buffer for bone after EDTA wash:

- 30 mM Tris-HCl (pH 8.0)
- 5 mM EDTA
- 50 mM NaCl

Lysis buffer / Digestive buffer for Hair:

Reagent	Volume
1 M Tris HCl (pH 7.5)	1 ml
0.5 M EDTA	2 ml
5 M NaCl	10 ml
20% SDS	10 ml
DW	86 ml

Note: Do not autoclave. Store at 40°C

ANNEXURE II

Protocol for solution preparation for gel electrophoresis

The TAE buffer is used as tank buffer and solvent for preparation of agarose gels during electrophoresis. Ethidium bromide is added to agarose gel before leaving it for solidifying. The 6X loading dye is used to dilute the sample to be loaded in the gel. The method for preparation of these solutions for use in agarose gel electrophoresis is given here:

Name of reagent	Name of chemicals used	Quantity	Volume prepared*
TAE buffer	Tris (hydroxymethyl) aminomethane NH2.C(C2OH)3 salt	121.00 g	500 ml
	0.5M EDTA (pH 8)	50 ml	
	Glacial acetic acid	28.55 ml	
6X Loading dye	Bromophenol Blue	0.125 g	50 ml
	Xylene Cyanol FF	0.125 g	
	Glycerol	15 ml	
Ethidium bromide solution	EtBr	10 mg	1 ml

***Volume made up with DW**

After adding the chemicals in a measuring cylinder, DW was added to make up the volume to the required volume. The pH of the solution was measured and maintained at 8.0.

ANNEXURE III

Reagents used for a typical Polymerase Chain Reaction (PCR), concentration required and their functions in brief

Reagent	**Stock concentration**	**Working concentration**	**Function**
Taq DNA polymerase	3 Units	1 Unit	Replication of DNA
Taq buffer	10X	1X	Maintains the optimum salt concentration and pH for *Taq* polymerase to function
dNTPs	200 µM	25 µM	Act as substrates for the polymerase; building blocks for DNA replication
MgCl2	15 mM	1.5-3 mM	Mg^{2+} acts as a co-factor for *Taq* polymerase; the concentration of Mg^{2+} regulates the specificity of the enzyme
Primers (Forward and Reverse)	100 picomoles	10 picomoles	Act as precursor for initiating replication
DNA Template	~ 400 ng/µl	25-40 ng/µl	Serves as the substrate for *Taq* DNA polymerase to bind

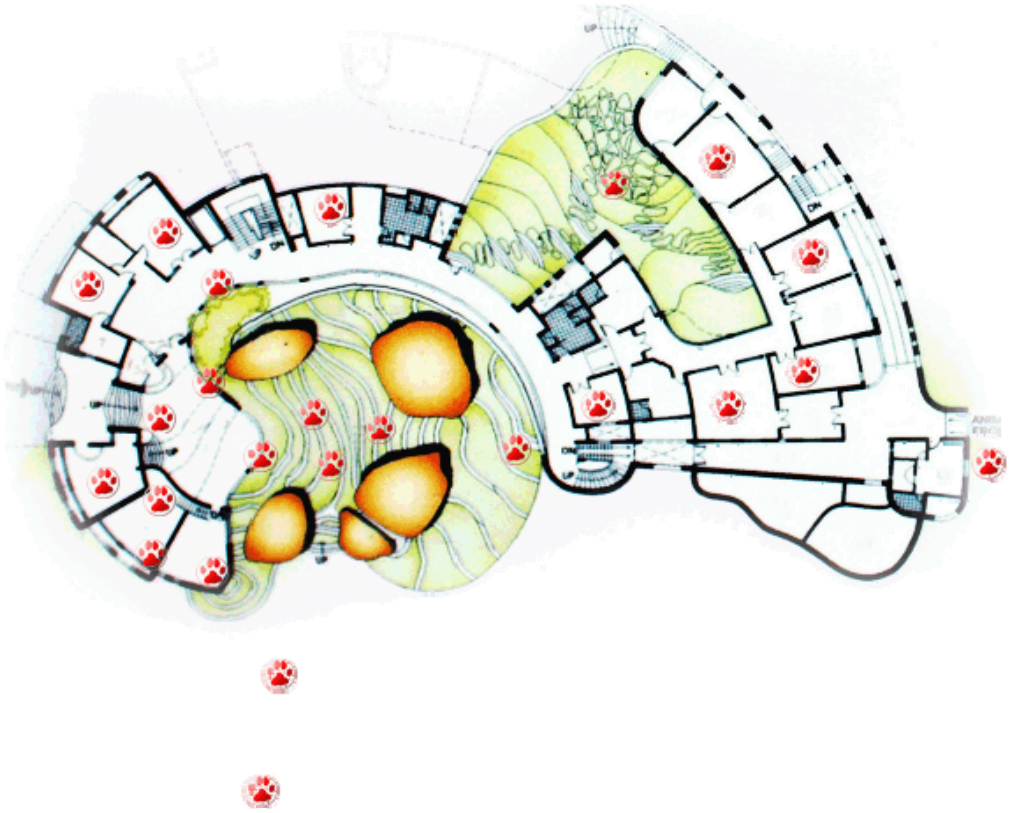
ANNEXURE IV

Softwares used for various DNA analyses

S No.	Name	Web Link
1	CodonCode	http://www.codoncode.com/
2	BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3	GeneMapper	https://www.thermofisher.com/order/catalog/product/4475073
4	CERVUS	http://www.fieldgenetics.com/pages/aboutCervus_Using.jsp
5	GenAlEx	http://biology-assets.anu.edu.au/GenAlEx/Welcome.html
6	ML – Relate	http://www.montana.edu/kalinowski/software/ml-relate/index.html
7	MEGA	http://www.megasoftware.net/

About CCMB LaCONES

Effective conservation measures include both *in situ* habitat preservation, species protection and *ex situ* conservation (captive breeding in controlled environment to restock original wild populations). LaCONES was established to support both these measures using biotechnological tools and techniques in an innovative manner. Project LaCONES was established in 1998 with support from Dept. of Biotechnology (DBT), Govt. of India, New Delhi, Central Zoo Authority of India (CZA), New Delhi, Council of Scientific and Industrial Research (CSIR), New Delhi and Government of Andhra Pradesh. The laboratory was later established in 2007. This lab has since then strived to promote excellence in conservation biotechnology and serve for the conservation of endangered wildlife in India.



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