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Wildlife forensic techniques: DNA Extraction from hard Biological matter ofChital (*Axis axis*), molecular analysis,use of SEM andEDXin species identification.

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Abstract: *India is globally recognised as Mega bio-diverse nation, encompassingplethoraof floral and faunal species within its numerous Eco-zones. This wildlife treasure is currently threatened to extinction by rampant poaching and illegal trade. DNA identification of species unravels potential possibilities in the field of conservation biology. The information, database on genetic diversity of wildlife is necessary to ascertaingenetically deteriorated populations in order to formulate better conservation prospects. Accurate identification of the threatened species not only provides understanding of theirmorphological characteristics but also the existing state of their vulnerability and prospects for their conservation. DNA based species identification is the most reliable and acceptable evidence in forensic investigations. In the current study the chital DNA was extracted from its hard biological matter (Bone, Meat) using Banglore GeNei Kit. It was observed that the Banglore GeNei kit could efficiently extract good quality of Chital's DNA i.e. 2.4mg/µl, 1.8mg/µl from meat and bone samples respectively. The molecular analysis through PCR amplification of the desired gene was conducted for accurate identification of species. The physical verification and element analysis was accomplished using SEM (Scanning electron microscope) and EDX (Energy Dispersive X-ray Microanalyser) on dorsal primary guard chital hair samples. The scanning electron micrographs of Chital showed distinct characteristic of circular pattern and EDX Generate spectra Cheetal-375.dot 1 that provided more localized 12 elemental composition of chital.*

Keywords: *Wild life, Forensic techniques, Biodiversity, conservation, Chital, DNA Extraction, PCR, SEM, EDX.*

I. Introduction

Wildlife DNA forensics is a discipline that has emerged from conservation genetic research and forensic genetic practice to meet the increasing need of investigative tools in wildlife protection. The population of majority of wildlife species isdeclining;some are endangered while few species are already extinct predominantly due to illegal hunting, poaching, habitat destruction and other factors. In order to keep a check on dwindling wildlife Species, majority of faunal diversity has been protected under different legislations at national as well as international level. In India, most of the species are protected under the Wildlife (Protection) Act, 1972 of India (WPA), which further includes different Schedules and articles based on level of crime committed. While species which are internationally traded, are monitored by regulations of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Wild life forensic techniques are consider important for the accurate identification of vulnerable species, providing viable scientific evidences for the establishment of standardised protocols for their conservation.

Identification of species from biological samples is an important aspect in wildlife forensics. Some of the evidences like Skin, hairs, antlersetc. when intact can be identified using simple microscopic and morphometric techniques and are cost effective. But sample if degraded or processed or other sample like, finished wildlife products, urine, saliva, formalin preserved samples, tanned skins, Hair, Boneetc. requires robust techniques over microscopy and morphology to identifying the species. (2, 3).

Spotted deer or commonly called as "Chital" are one of the most common wild herbivore found in forests of India. The chital deer (Axis axis), also called the Indian spotted deer and the Axis deer, is native to India and Sri Lanka. Being so ubiquitous this deer species can act as a model organism for conservation genetic as well as wildlife forensic studies. Over the last decade there has been an increased requirement for the isolation of pure genomic DNA that performs well in any downstream application. Such downstream application includes Polymerase Chain Reaction (multiplex PCR, real time PCR etc.).Southern blotting, Amplified fragment

length polymorphism, Random amplification polymorphic DNA length, Micro satellite analysis's techniques. For theseanalysis techniques, DNA quality is the single most important factor. Poor quality DNA can lead to sub optional result and DNA that is impure or contaminated will not perform well in various applications.

DNA is a relatively stable molecule. However introduction of enzymatically active nucleases to DNA solution should be avoided as these enzymes will degrade DNA. DNA is subject to acid hydrolysis when started in water and should therefore be started at a slightly alkaline pH e.g. in TE buffer or in buffer AE from QIAGEN.Degration of DNA has a major effect on any result obtained generating errors that are both quantitative and qualitative for example, a reduction in DNA size may lead to the failure of downstream application such as PCR based applications and Hybridization. This is especially important in areas where DNA degradation is common phenomenon in the original sample. (**Faerman,M., Filon,D., Kahila,G., Greenblatt,C.L., Smith,P. and Oppenheim,A. 1995 *Gene*, 167, 327–332.[ISI][Medline](10)**.DNA extraction by the various types of the technique is the most important in the field of forensic science with the help of these techniques we can know the purity and quality of DNA which is present in the tissue, bones, skin of the animals. Forensic science is basically depending on the study of DNA.

SEM& EDX techniques are found to be very useful in identification of species through hair. There is very little work has been done on EDX. Which is still to be explored to find out its utility and significance in identification of the species?

The present study aims at DNA extraction from the bone sample of Chital which are collected during the field survey from the Rajaji National park Rishikesh, Uttarakhand. DNA extracted with the help of commercially available Bangalore GeNei kit and the PCR amplification of extracted DNA. Analysis by using SEM and EDX instruments.

II. Material and Methodology

2.1-Sample collection: The bone and hair samples were collected from extensive field survey conducted in Rajaji National park, Jim Corbett National park, and Chila mountain range of Uttarakhand India in 2013-14.Hundred collective samples of hair and bones of chital (*Axis axis*) were collected and preserved in formalin solution at -20ºC for further laboratory analysis. DNA isolation was carried out within 7-10 days to avoid degradation of DNA quality.

Fig.1-Bone samples

Fig.2-Banglore GeNei Kit.

2.2-DNA isolation-In wildlife forensic, extraction of genomic DNA from the Bone sample is very typical and most important step for the case sample. Only the successful procedure of DNA isolation along withthe modifications that were carried out and purification is reported here. To carry out the genomic DNA isolation, the Bangalore GeNei kit (Bangalore Genei, 2007) was used.

2.3-Solutions and solvents

(A)Deionized water
(B) 0.5% sodium hypochlorite.
(C) 0.5ml. EDTA (pH. 8.0),
(D)50 µl proteinase K.
(E)Chloroform: Iso Amyl alcohol (24:1).
(F)Isopropanol, Ethanol 70% and 96%.
(G) Solution A and solution B as per the prescribed protocol of the kit.

2.4-Standard Procedure:

- The soft tissue were removed completely form fresh /boiled boneand bone marrow was removed using sterile razor blades.
- Bone samples were first washed with 0.5% sodium hypochlorite and then Rinsed for 5 minutes with deionised water.
- Bone sample were air dried and exposed to UV irradiation for one hour.The bone was drilled or scraped to make powder using (*make bone powder*).
- 250 mg of the bone powder was taken into sterile 15 ml polypropylene tube. 2 ml of 0.5ml EDTA (Ph. 8.0) was added and placed on tube on a rocking platform over night at room temperature (well preserved material requires 2-3 days forcomplete decalcification).
- Centrifuged at 5000 rpm for 10 min and discard the supernatant. Re-suspend the pellet in deionised water. Centrifuged at 5000rpm for 10min and discard the supernatant.
- 600 µl of solution A (Ensure that 50 µl proteinase K has been added to solution A) was added and kept at 50°C for 24-48 hrs. with gentle rocking or in a water bath at 50°C with regular shaking and mixing after every 3-4 hours for 10 min .(Optional : Keeping longer for 2-3 days will ensure better lysis).
- 250 µl solution B was added and mix thoroughly for 1 min and then centrifuged at 5000 rpm for 10 min at RT.The supernatant was transferred into new 2 ml centrifuge tube and add 350 µl of solution C and mix gently for at least 10 min.
- The spin column in 2 ml collection tube (provided). The supernatant was passed through the spin column, 600 µl each time .
- Then Centrifuged at 8000 rpm for 1 min.After each spin, discarded the flow-through; placed the column in same collection tube before proceeding with the next spin.Column was washed with 500 µl of wash buffer I, and centrifuged at 8000 rpm for 1 min, discard flow-through and place the column in same collection tube.
- Column was washed with 700 µl wash buffer II and centrifuged at 13000 rpm for 3 min. discarded flow – through and placed the column in same collection tube before proceeding with next spin.
- A final spin was given for 2 min at maximum speed. (This would ensure removal of alcohol from column).The column was placed in new 1.5 ml micro centrifuge tube eluted DNA with 50-100 µl (10-20µl for boiled and ancient bone) elution buffer .Incubated at room temperature for 2 min , Centrifuged at 10000 rpm for 1 min.

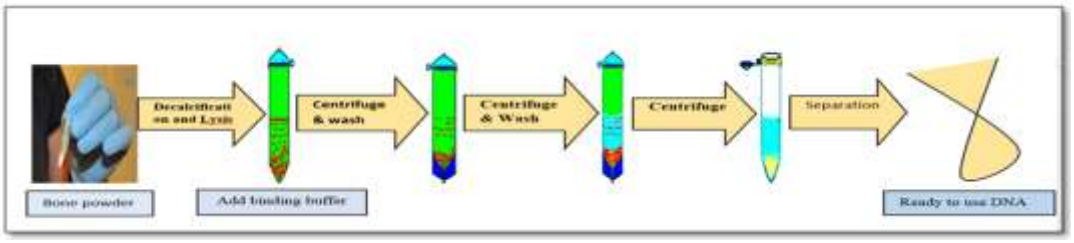

Fig-3: Flow chart of DNA extraction.

PCR amplifications integral part of wild life forensic techniques responsible for amplification of DNA. With this technique the DNA bands and sequencing of the DNA is visualised to identify the species in more efficient manner. The PCR cycle consisted of following steps: initial denaturation at 94°C for 5min, followed by 32 cycles of denaturation at 94°C for 35 seconds, primer annealing at 53°C for 45 seconds, primer extension at 72°C for 35 seconds, and a final extension at 72°C for 10 min.The PCR products obtained were then subjected to electrophoresis on 2% agarose gel and visualized over an UV trans-illuminator to assess amplification. Duplicates, negative and positive controls were performed in DNA extractions and PCR amplifications.

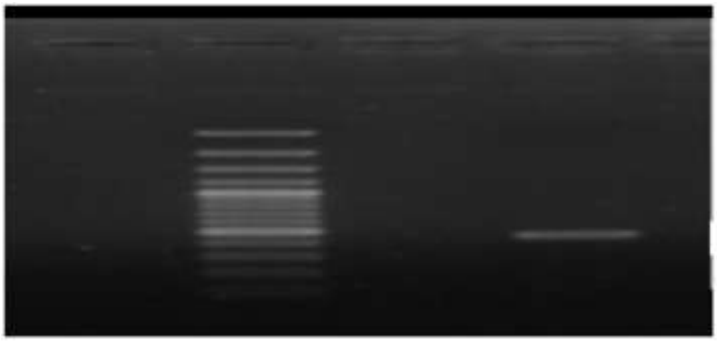

Fig-4 Analysis of the PCR amplicon in 1% agarose gel electrophoresis, containing 0.5 μg/mL ethidiumbromide. LaneM: 100 bp plus DNA ladder, Lane NTC: no template control, and Lane

Sequencing.PCR forward primer (Cytb 381, L-5'GCCCCTCAGAATGATATTTGTCCTCA-3'), and (12S rRNA Fwd 5' AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3) gene wereused for the sequencing reactions using the Big Dye® Terminatorv3.1 Cycle Sequencing kit (Applied Biosystem). Sequencing reactionproducts were purified using a standard ethanol-isopropanolprecipitation method and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystem, USA).

2.5-SEM study and EDX: Dorsal primary guard hair were selected for SEM and EDX studies The samples were processed in alcohol series 50%, 70%, 100% ethyl alcohol (each for five minutes). Then they were kept in acetone for 15 minutes for removing the dust particles. The cleaning of hair was repeated three times in acetone for 15 minutes in sonicator. The cleaned hair was kept on tap present on the stubs. The coating of hair was done with carbon by using coating chamber, and the stubs were used for SEM, EDX analysis.

SEM **Coated Stubs** **Carbon Coating unit**

Fig-5: Samples preparation for SEM and EDX analysis.

III. Result and Discussion

The study reveals that the good quality of DNA was extracted using Bangalore GeNei kit. The quality and purity of DNA extracted was assessed using **Bio photometer** AV223331.Cyt b gene (Cytochrome-b) was effectively used for the identification of species in degraded samples as well as formalin preserved samples. Chital species were identified using molecular forensics techniques. The efficient extraction of DNA from meat and bone samples of chital was 2.4mg//µl and 1.8mg/µl respectively.

Table No.1: Quantity of DNA extracted

Animal used	SAMPLE	Reading
CHITAL	MEAT	2.4mg/µl
CHITAL	BONE	1.8mg/µl/

PCR amplificationof DNA. With this technique the DNA bands and sequencing of the DNA is visualised to identify the species in more efficient manner. A fragment of the12S rRNA gene (*Ca*. 420bp) and Cytb gene (*Ca*.350bp) were amplified using the universal PCR primers (Kocher et al.1989; Mayer, et al., 1995) (7, 8). All PCR reactions were carried out on Applied Bio systems 2720 Thermal Cycler in a total reaction volume of 20 µl containing 1x PCR buffer; 25 mM MgCl2, 10 mM dNTPs, 10 µM of each primer, 2.5 U Taq

polymerase (MBI, Fermentas) and 2 µl of total DNA. In gel electrophoresis the Bands of Chital DNA are clearly visible, which efficiently help in identifying the species.

Table 2. Similarities in the Cytb locus b and sequences of most similar deer species. Hog deer, Chital and Swamp deer available in GenBank.

Specimen	**Query coverage (%)**	**Similarity (%)**
Hog deer (*Axis porcinus*)	100	100
Chital (*Axis axis*)	100	100
Swamp deer (*Rucervus duvaucelii*)	100	100

3.1-SEM ANALYSIS: The study of hair cuticular pattern is found to be useful in identification of the species for ecological studies & also for providing scientific proof for WILDLIFE FORENSIC cases **Bahuguna, A. 2008 (14)**. The scanning electron microscopy and Energy Dispersive X-ray Microanalyser play a significant role in the forensic technology era for species identification and conservation. Future trend relies on use of universal primers that can produce amplification of spectrum of species and ultimately detect Forensically Informative Nucleotide Sequences (FINS) in closely related organisms. Sagacious use of Molecular Forensic techniques would help enforcement agencies for effectively controlling poaching and in better wildlife conservation. The study revealed that the technique is useful for distinguishing the species. The SEM is very useful instrument for the science & especially for forensic science many type of clue in case of crime such as hair, skin part, finger print can be easily seen by SEM.

Table-3: The scanning electron micrographs of CHITAL showed distinct characteristic of cuticular patterna.

Species	Cuticular pattern	Scale pattern	Scale margin	Distance between scales	
Chital	Hair part	SP	SM Crenate	DS	
(*Axis axis*)	Medial	Regular		Near	5.0± 0

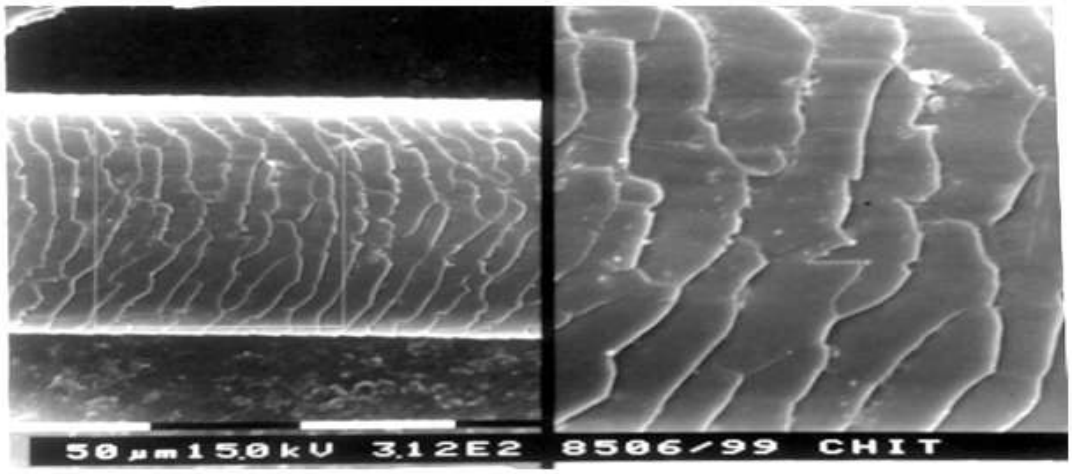

Fig.6- scanning electron microscopic images of chital hair.

3.2-EDX ANALYSIS-(Energy Dispersive X-ray Microanalyseris based on the photon nature of light. In the X-ray range the energy of a single photon is just sufficient to produce a measurable voltage pulse as the output of an ultra-low noise preamplifier connected to the low noise is a statistical measure of the corresponding quantum energy. By digitally recording and counting a great number of such pulses within a so called multi-channel analyzer (MCA), a complete image of the X-ray spectrum builds up almost simultaneously. This digital quantum counting technique makes the Energy Dispersive X-ray Microanalyser exceedingly reliable. It is a useful tool instrument in the identification of species. The technique provides the composition of various elements in hair strands It facilitates the analysis and comparison of the elemental degradation in the species due to various factors over time.

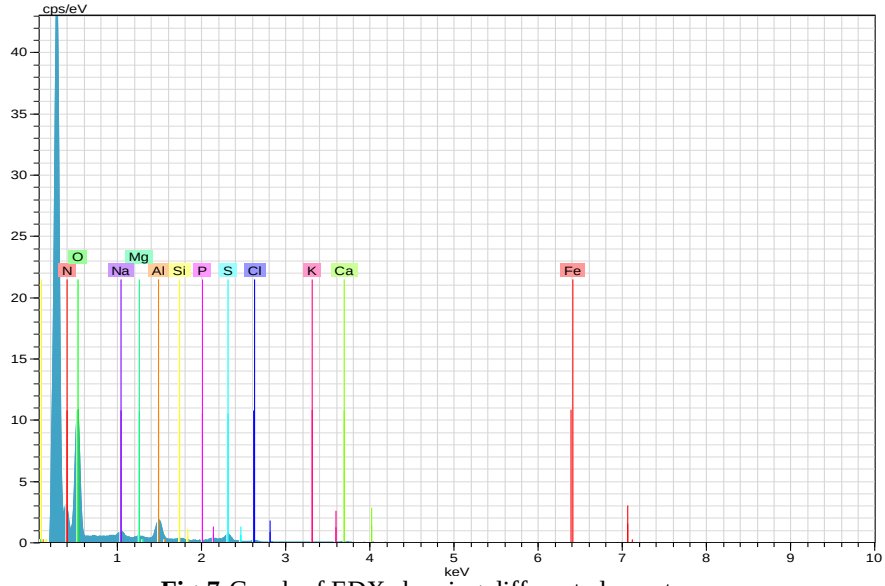

Fig-7: Graph of EDX showing different element.

Table-4: showing elements inEDX study of Chital hair strand.

ElAN	Series	unn. (Wt -%)	C norm. [wt.-%]	C Atom. [at.-%]	C Error [%]
Al	13 K-series	2.92	2.92	1.80	0.2
Na	11 K-series	2.62	2.62	1.90	0.2
S	16 K-series	1.48	1.48	0.77	0.1
Mg	12 K-series	0.86	0.86	0.59	0.1
Ca	20 K-series	0.77	0.77	0.32	0.3
P	15 K-series	0.40	0.40	0.21	0.1
Si	14 K-series	0.29	0.29	0.17	0.1
Fe	26 K-series	0.21	0.21	0.06	0.2
Cl	17 K-series	0.06	0.06	0.03	0.1
N	7 K-series	0.00	0.00	0.01	0.1
K	19 K-series	0.00	0.00	0.00	0.0
O	8 K-series	90.39	90.39	94.14	28.5

Table -5: Details of used species and GenBank Accretions number of used Cytochrome b and 12S ribosomal RNA.

Species	**Common name**	**Gene Bank Accession**	**Number**
Axis axis	Chital	JN093090.1,N093092.1	JN093077.1, JN093073.1
Axis porcinus	Hog deer	EF579805.1,EF491204.1	AY775785.1AY184435.1
Cervus unicolor	Sambar	JN861032.1	
Rucervus duvaucelii	Swamp deer	EF079830.1	EU908275.1, EU084669.1
Muntiacus muntjak	Barking deer	JQ991600.1	AF294731.1
Antelope cervicapra	Black buck	AF022058.1	
Tetracerus quadricornis	Four horned antelope	AF036274.1	EF175739.1
Gazella bennettii	Chinkara	N410357.1	EF133853.1
Bos gaurus	Gaur	AB077316.1	EF219403.1
Panthera tigris	Tiger	KC879297.1	AY736583.1

IV. Conclusion

Ungulate species mainly dears, rhinoceros etc. are in great demand and remains soft target for poachers mainly because of their growing demand for meat, skin, ornamental purpose, medical uses and other local commercial purposes. The accurate identification of the vulnerable species remains vital for their future conservation and protection. The current study highlights the effective DNA isolation technique and Molecular identification technique by using SEM and EDX, which helped in accurate identification of required species Chital. Bangalore genie kit proved to be highly productive in sufficient extraction of DNA from hard biological matter of Chital. EDX technique identified 12 major elements found in hair strand of Chital. These techniques have proved to be forte in wildlife forensic analysis and Future trend relies on use of universal primers that can produce amplification of wide spectrum of species.

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