CHARACTERIZATION OF BONE, RHINO HORN AND ANTLER FOR IDENTIFYING SPECIES TO DEAL WILDLIFE OFFENCES

THESIS
SUBMITTED TO THE
FOREST RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
DEHRADUN, UTTARAKHAND
For
THE AWARD OF THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN FORESTRY
(WILDLIFE SCIENCE)

By

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Wildlife Institute of India,
Chandrabani, Dehradun,
Uttarakhand, India

2007

DECLARATION

I hereby declare that the thesis entitled **"Characterization of bone, rhino horn and antler for identifying species to deal wildlife offences"** submitted to Forest Research Institute (Deemed University), Dehradun, for the award of the degree of Doctor of Philosophy in Wildlife Science, is a record of original research work done by me under the supervision of Dr. S. P. Goyal, Scientist 'SF', Wildlife Institute of India, Dehradun, and it has not formed the basis for the award of any other degree or diploma. I also declare that the thesis embodies my own work, observation and analysis and in that respects the investigation appears to advance knowledge in the subject.

Date: 23rd February, 2007
Place: Dehradun

Rina Rani Singh

(Rina Rani Singh)

Candidate

Countersigned:

(Dr. S. P. Goyal)
Supervisor

S. P. Goyal, Ph.D
Scientist 'SF' and Nodal Officer,
Wildlife Forensic Cell

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

CERTIFICATE

This is to certify that the thesis entitled **"Characterization of bone, rhino horn and antler for identifying species to deal wildlife offences"** submitted for the award of the degree of Doctor of Philosophy in Wildlife Science to Forest Research Institute (Deemed University), Dehradun, Uttarakhand, India, contains original research work carried out by Smt. Rina Rani Singh under my guidance and supervision. No part of this thesis has been submitted for any other degree and it fulfills all the requirements laid down in the Ordinance of Forest Research Institute (Deemed University) Dehradun for this purpose.

Place: Dehradun
Dated: 23rd February, 2007

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Appendix -3

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(S.D. ARORA)
Registrar
F.R.I. Deemed University

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1. Dr. S.P. Goyal, Scientist-SF, Wildlife Institute of India, P.B. No. 18, Chandrabani, Dehra Dun – 248 001.
2. The Nodal Officer, Wildlife Institute of India, P.B. No. 18, Chandrabani, Dehra Dun – 248 001.
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Yours faithfully,

(S.D. ARORA)
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Contents

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Summary

Characterization of bones, rhinoceros horn and antler to identify species are very important since there has been global concern to check the illegal trade to at least keep it to the sustainable level. India being one of the important biodiversity nation has yet greater task to protect their flora and fauna. Concern animals possessing these parts always have threat of poaching. The annual international trade in wildlife and its products has been estimated to be of approximately 20 billion USD, and the gravity of the illegal trade in wildlife alone can be judged by the economic value that is worth 5 billion USD, which in economic terms ranks second after the drugs. Stringent international and national laws to protect these natural resources even then sometime it becomes difficult to prove offences in the court of law due to non-availability of proper evidences. In such circumstances, characterization of the above mentioned animal parts are of utmost importance, as these have widely been reported in wildlife offences.

Species for this Ph.D. study were selected according to the endangered status of the species and since these products were difficult to identify mostly in processed form. Therefore, attempts were made to characterize bones of tiger (*Panthera tigris*) and leopard (*Panthera pardus*), greater one horned rhinoceros (*rhinoceros unicornis)* horn and antlers of chital (*Axis axis*), sambar (*Cervus unicolor*), swamp deer (*Cervus duvauceli*). Other than these species mentioned in synopsis, antlers of hog deer (*Axis porcinus*) and barking deer (*Muntiacus muntajak*) and ivory of Asian elephant (*Elephas maximus*) have also been characterized for comparison purposes.

The present study is an effort to develop methods to characterize these items for conservation of the concern species and objectives of this study are as follows:

1. Determine morphological, crystallographic (XRD & XRF) and DNA characteristics of major bones of tiger and leopard, and
2. Determine morphological, crystallographic (XRD & XRF) and DNA characteristics of rhino horn and antler of chital, sambar and swamp deer species.

Different techniques were used to characterize the above mentioned animal products so that even if it is transformed into powders, that can be identified using its characteristics. In this study, morphological features have been used to differentiate bones, horns and antlers. These biological materials have its unique characteristics. But, when they are found in processed form, loses their morphological features. Hence, some characteristics were needed to identify these materials in processed form. Many of analytical techniques were attempted to characterize processed biological materials. Use of combination of techniques increases the accuracy of identification and help in confirmation of the result. Scanning electron microscopy is one of the techniques used to study the surface morphology of any object. This technique was used to examine differences in the surface topography of biological materials. X-ray diffraction was used to know the crystalline structure of these wildlife products and to differentiate it from similar products. X-ray fluorescence an, instrumental technique was used to know the qualitative elemental composition. Further, the quantitative analysis was done using inductively coupled plasma – mass spectrometry. Thermo gravimetric technique was used to examine the nature of weight loss with constant increase in temperature. Protein profile was used only for rhinoceros horn. DNA analysis was performed for all these biological materials.

Chapter 1 is titled "Characterization of bone, rhino horn and antler for identifying species to deal offence cases: a need for conservation" contains an account of wildlife trade, need for characterization, species in study, objectives, hypothesis, methods used, analysis at different Institutions, usefulness of this study, organization of the thesis, limitation of this study and references.

Chapter 2 is titled "Characterization of tiger and leopard bones" contains brief information on illegal trade of tiger and leopard bones, details of literature review and methodology used for characterization of tiger and leopard bones, result, discussion and references. Through morphometry, major bones (skull, scapula, pelvic, femur and humerus) of tiger and leopards were successfully differentiated. Difference in surface topography at three areas viz. outer, cortex and core of tiger and leopard bones were examined using scanning electron microscopy. Core portion was most suitable to

differentiate tiger and leopard bones. X-ray diffraction was used for powdered samples to know the differences in crystal structure but, however differences were not noticed. Thermo gravimetric analysis was successful in differentiating tiger and leopard bones. The rate of weight loss was found different at first peak in leopard (0.7%/min at 56 ^{0} C) and tiger (1.2%/min at 58 ^{0} C). The rate of weight loss at the second peak in tiger was twice (3.8%/min at 329 ^{0} C) than the leopard (1.9%/min at 328 ^{0} C). The water loss percentage was also different in tiger (approx 8%) and leopard (5%). Differences were also noticed in weight left at 1400 ^{0} C in leopard (70.30%) and tiger (56.55%). This method could successfully differentiate powdered samples of bones.

X-ray fluorescence indicates that intensity of chlorine, sodium, aluminum and iron can be used to distinguish tiger and leopard bones. Elemental intensity concentration was determined using inductively coupled plasma – mass spectrometry. Discriminant function analysis revealed that a function comprising of calcium (Ca), copper (Cu) and sodium (Na) concentrations could classify both bones with 100% accuracy. DNA (300-400bp) was extracted from bones of tiger and leopard using Gene clean and Bio Robotic methods. Successful amplification of PCR product of DNA samples was achieved using primer cytochrome b and 16S rRNA genes. Further DNA sequencing was done. Alignment of tiger and leopard sequences revealed more than 20% polymorphism in cytochrome b region at 69 different sites out of 319bp and 6.35% polymorphism in 16S rRNA at 23 different sites out of 362 bp. Forty-two and 14 restriction enzymes at cytochrome b region were specific to tiger and leopard DNA respectively and 22 restriction enzymes were common and cut the DNA sequence at different positions. Thus, these restriction enzymes can be used to differentiate tiger and leopard species. Using restriction enzymes which were cutting the 16S rRNA gene fragment at different sites in tiger and leopard, it was found that 7 restriction enzymes were specific to tiger DNA fragments where as 10 restriction enzymes to leopard DNA sequences and 20 restriction enzymes common to both tiger and leopard sequences cuts at variable positions. Further, universal primer for both tiger and leopard was developed using software Primer 3 of fragment length of 167-177 bp for cytochrome b and 258-285 bp for 16S rRNA gene to amplify smaller fragments which

were extracted from bones. After amplification with these primers, sequencing can be accomplished to differentiate tiger and leopard bones.

Chapter 3 is titled "Characterization of rhinoceros horn (*rhinoceros unicornis*)". This chapter deals with introduction of rhinoceros species, illegal trade of rhinoceros horn, literature review, methods of characterization of rhinoceros horn, result, discussion and references. Morphological characteristic of horn viz., presence of tubercles, grooves and bulge gives clue to identify rhinoceros horn. Discriminate function analysis of the quantitative measurements related to morphometry could differentiate rhinoceros horn and buffalo horn with 100% classification accuracy using two functions. Rhinoceros and buffalo horn had different densities 7.49 g/cm^3 and 4.54 g/cm^3 respectively. Mann-Whitney U test for densities were $p < 0.0001$ and asymptotic significance (2-tailed) were $p < 0.025$. The scan electron micrographs of rhinoceros horn showed numerous pores on the ventral side, each pore had several sub-pores of 9.39 μm (range = 5.56 to 10.11 μm) diameter. This is a remarkable characteristic of rhinoceros horn and can be potentially used for its identification. The buffalo horn micrographs of dorsal surface does neither indicates presence of hairs nor pores on the ventral surface, instead there were presence of twisted fibers which had dark zone in the centre. X-ray diffraction (XRD) of rhinoceros horn indicates typical characteristic having minute peaks at 26^0, 43^0 and 50^0 which were not noted in buffalo diffractogram. Background intensity of rhinoceros horn was higher as compared to buffalo horn. Two suspected cases of rhinoceros horn were analyzed using XRD and compared with the reference diffractogram and both the samples turned to be fakes. Thus, these characteristic peaks can be used for identification purpose and the specificity of the diffraction pattern can distinguish the rhinoceros horn efficiently from other horns. A characteristic exothermic peak in thermograph of rhinoceros horn in between 200 to 400 ^{0}C would be useful in identification of rhinoceros horn products.

The qualitative elemental fluorescence obtained from rhinoceros horn reveal that out of the fourteen elements analyzed, the intensity (KCps) of iron (Fe) was highest where as intensity of magnesium (Mg) was lowest. ICP-MS analysis of Asian

rhinoceros horn revealed that, calcium (Ca=67%) is the most abundant element, followed by Fe=12%, Na=10%, Mg=4%, K = 4%, Co=2% and Zn=1% with other elements in traces. Three elements viz. Mo, U and Cd were however not detected. However, mean concentration of four elements Ca, Na, V and Pb were different in wild and the zoo rhinoceros horns. The rhinoceros horns from zoo have higher concentrations of all four elements and the observed difference was almost thrice except calcium (Ca). The difference in calcium (Ca) and sodium (Na) concentrations are infer to be due to difference in feeding habits and high vanadium (V) and lead (Pb) concentrations reveals that the atmosphere of in wild is less polluted than zoo in metropolitan city. Protein profile analysis through sodium dodicyl sulphate – polyacryilamide gel electrophoresis reveals variation in number of protein bands and their positions in case of rhinoceros horn and buffalo horn. Based on the molecular weight calculated a dendrogram was generated using Ward's method to cluster rhinoceros horn and buffalo horn which clearly differentiate rhinoceros horns from buffalo horn based on presence of protein bands at different positions.

DNA was extracted from rhinoceros horns using Qiagen protocol and it yielded 150 to 600 bp of DNA and successfully amplified with cytochrome b (450 bp) and 16S rRNA (550 bp) genes of mt-DNA. Alignment of *Rhinoceros unicornis, Bos taurus* and *Bubalus bubalis* sequences revealed 24.65% polymorphism with 90 different sites out of 365bp of cytochrome b gene of mt-DNA. Five restriction enzymes are specific to *Rhinoceros unicornis* where as 15 restriction enzymes were specific to *Bos taurus* and 49 to *Bubalus bubalis.* None restriction enzyme were common to all three species at cytochrome b region. Thus, specific restriction enzymes for these three species can be used to differentiate them. Further, universal primer for all species was developed using software Primer 3 of fragment length of 210-250 bp for cytochrome b gene to amplify smaller fragments which were extracted from horns. After amplification with these primers, sequencing can be accomplished to differentiate these three species.

Chapter 4 is titled "Characterization of antlers" deals with the introduction to the antlers of different species, trade of antlers, literature review on antlers, methods

for characterization, results, discussion and references. Antlers of different deer species can be differentiated based on their morphological features viz., branching patterns, numbers of tines, curvature, angles and roughness. Number of tines was specific to species and helpful in distinguishing antlers of different species. Antler of swamp deer had more than two tines, barking deer only one tine where as others chital, sambar and hog deer had two tines each. Only second tine of hog deer points downward and other tines of all the other four deer species and first tine of hog deer points upward. The above feature of hog deer antler can be diagnostic for its antler and can differentiate it from antlers of chital and sambar. Among chital and sambar antlers, the dichotomy of second tine of chital antler occurs at different portion of main beam and this feature can be used to differentiate antlers of chital and sambar. The number of angles depends on the presence of number of tines. The first angle indicate that swamp deer antler has widest mean angle measurement (109.25±1.87), followed by chital (103.17±1.66), sambar (65.59±1.87), whereas hog deer and barking deer antlers had overlapping mean angle measurements. The barking deer antler does not have second tines and angle. Second mean angle measurements were observed to be widest in swamp deer antler (106.20±2.97) followed by hog deer (85.26±2.18), sambar (58.12±3.11) and chital (54.35±2.06). The branching pattern can substantiate wherever there is overlaps in the angle measurements. Data indicates that first angles can be used to differentiate antlers of various species. High overlap in the second angle measurements was noted in chital, sambar and hog deer except for swamp deer. Thus, this alone cannot be used to differentiate antler of different species, but corroborating with morphological features may allow distinguishing species. The angle measurements of first versus second angles were plotted, however few overlaps were noticed. These might be due to the age effect. These overlaps can be corrected by using physical parameters like branching patterns and number of tines etc. Discriminant Function analysis of the quantitative variables of morphometric measurements was also useful in differentiating antler of different species. Three functions derived using ten variables could absolutely differentiate the antlers of various species using Discriminant Function Analysis (DFA). First function could itself explain 57.3% variability. Canonical Discriminant function could cluster antler of different species with few overlaps due to antlers of various age groups. In such cases other analytical techniques can be used for inter-specific distinction of antlers.

Differentiation in cross-sections of antler of various species can be used for identification.

Scanning electron microscopy (SEM) can also be used successfully to differentiate species from antler and the surface topography of core portion gave best distinction. All antler samples matched with hydroxyapatite minerals (American Standard Test Matching file no. 9, card no. – 432). Since ivory is also hydroxyapatite it was compared with antlers and a minute but distinct difference was noticed. Diffractogram of antler showed hump after 50^0 whereas in ivory a slanting line was observed at this position. Crystallite size of ivory ranged between ~ 16.9 and 86.1 nm where as antler ranged between ~ 26.5 and 169.8 nm. Cell parameter 'a', 'c' and cell volume in case of antler (11.70, 6.92 and 820.22) was higher than ivory (9.49, 6.87 and 535.80), cell parameter b = a thus, not measured separately. There were minute differences in the diffractogram of antlers of different deer species but, were not consistent. Dendogram generated based on the presence and absence of diffraction peaks could cluster separately the antlers of different species.

Thermo gravimetric analysis indicates maximum losses were noticed in antlers of all species between 200 and 400^0C. Weight loss pattern of antlers of different species showed remarkable differences in three temperature sections, 0-300^0C, 450-700^0C and 1000-1400^0C. Weight loss in sambar antler was very distinct from other species in all three temperature sections. Data analysis of antlers indicates variation in percent water loss at 100^0C and the percent water loss pattern was highest in chital (7.5%) and lowest in antler of swamp deer (2.5%). Total percent weight left after burning till 1400 ^{0}C was highest in antlers of hog deer (58%) and lowest in barking deer (43.1%). Endothermic peak at 770-810 ^{0}C was only noticed in antler of chital. The antler of sambar had three consecutive exothermic peaks at 884 ^{0}C, 1120 ^{0}C and 1339 ^{0}C. The antler of swamp deer had two peaks between 800 ^{0}C and 1135 ^{0}C. Thermograph of hog deer and barking deer were quite similar except a minute exothermic peak at 150 ^{0}C in barking deer. These minute distinct differences can be successfully used as species specific signature.

The qualitative elemental fluorescence X-ray scan obtained from antlers of five species reveal that the intensity (KCps) of calcium, phosphorous, sulphur, chlorine, aluminum, zinc, potassium, chromium, manganese, and gadolinium were variable among the deer species and the intensities of magnesium, copper, nickel, and osmium had very slight differences. Intensity of strontium was different in antlers of hog deer, sambar and chital and was absent in antler of barking deer and swamp deer. Chital antler can be distinguished in having highest intensity of sulphur. Sambar antler had high silicon and zinc intensities and low iron intensity. Swamp deer antler had high chromium intensity. Barking deer had high potassium intensities. Inductively coupled plasma – mass spectrometry was used to know the quantitative elemental intensities in the antlers of different species. Two elements cadmium (Cd) and uranium (U) were not detected in antlers. Discriminant function analysis of the mean concentrations of elements could classify different antlers with 100% accuracy using four functions comprising of zinc (Zn), iron (Fe), calcium (Ca), potassium (K), magnesium (Mg), lead (Pb), sodium (Na), vanadium (V), nickel (Ni), copper (Cu), cobalt (Co), chromium (Cr), barium (Ba), strontium (Sr) and molybdenum (Mo). In respect of the reference levels of 5 ppm barium (Ba) and 52 ppm strontium (Sr) in bone matrix (Anon. 1980; Pais and Benton 1997), the mean levels of Ba could be regarded as 'elevated' in different species viz. chital (33.03 ppm), sambar (128.57 ppm), swamp deer (207.55 ppm), hog deer (55.42 ppm) and barking deer (143.86 ppm). Strontium level was also found to be elevated in antler of all species e.g. chital (90.18 ppm), sambar (64.79 ppm), swamp deer (71.79 ppm), hog deer (66.34 ppm), and barking deer (289.71 ppm) compared to the reference. Analysis of plants and soil will be needed to check the level of barium and strontium in the surrounding.

In present study, success was achieved in extraction of DNA from outer and core region of antlers. It is possible to extract DNA from other regions too but this needs standardization. PCR was successful with cytochrome b (186 bp) but, better amplification may be possible with more standardization. Alignment of *Axis axis*, *Cervus unicolor* and *Cervus duvauceli* sequences revealed that 89 different sites were observed out of 311 bp with 28.62% polymorphism in cytochrome b gene where as alignment of these three species DNA sequences revealed that 26 different sites were observed out of 532 bp with 4.89% polymorphism in 16S rRNA gene. Eight

restriction enzymes were specific to *Axis axis* fragment where as 5 restriction enzymes were specific to *Cervus unicolor* and 30 to *Cervus duvauceli*. 9 restriction enzymes which were common to all the three species, but cuts at variable positions. Seventeen restriction enzymes are specific to *Axis axis,* with no restriction enzymes specific to *Cervus unicolor* and only one restriction enzyme is specific to *Cervus duvauceli.* 21 restriction enzymes are common to all three species but, cuts at variable positions at 16S rRNA region. Thus, these restriction enzymes can be used to differentiate these three species. Further, universal primer for all species was developed using software Primer 3 of fragment length of 249-250 bp for cytochrome b gene and 234-249bp primer fragments for 16S rRNA. After amplification with these primers, sequencing can be accomplished to differentiate these three species.

Study has clearly revealed that the combination of different techniques can be used to differentiate species from different parts and products of tiger and leopard bones, Indian rhinoceros horn and antlers of different species.

At the end of the thesis a copy of the paper published in the Forensic Science International titled "Using morphometric and analytical techniques to characterize elephant ivory" and an extended abstract titled "Application of X-ray diffraction technique to characterize ivory, antler and rhino horn and its implication in wildlife forensic" has been attached.

CHAPTER 1

Characterization of wildlife products for identifying species to deal wildlife offence cases: a need for conservation

1.1. Introduction

India being one of the twelve identified mega-biodiversity nations is rich in flora and fauna and represents 8% of the total world's biodiversity (Dey 1996). It has 60% of the world's tiger, 50% of the Asian elephants, 70% of the Asian rhinos and the only country with Asiatic lion population in wild (Dey 1996). But, conservation of wild species today is a major challenge due to persistent illegal trade in wildlife parts & products (Mukherjee 1996). The annual international trade in wildlife and its products has been estimated to be of approximately US $ 20 billion, of which illegal trade in wildlife alone is of US $ 5 billion (Dey 1996) and in economic terms it ranks second after the drugs. Among mammals of India, trade in carnivores is more compared to herbivores (Anon. 1996). Along with trades in live animals, it also exists in parts and products. Major trade exists in skin (62%), ivory (7%), horn (3%), antler (2%), bone (11%), live animals (2%), feathers (1%), nails and claws (1%) and pod (1%) (Anon.1996). The availability of data on wildlife trade is just a tip of an iceberg but, there are lot more illegal trade cases that go unnoticed due to lack of awareness and constraint of enforcement agencies.

Over the years, poaching has increased which has depleted the population of several wild species and has brought them to critical level. Though appropriate protection has been given through CITES and *Wildlife (Protection) Act,* 1972, still lot more required to be done for their conservation. The *Wildlife (Protection) Act*, 1972 and its subsequent amendments in 1986, 1991 and 2003 have been instrumental in reducing the wildlife trade in India. But major loop holes are in characterization and identification of the confiscated wildlife parts and products, devoid of which conviction rate is very low (Anon. 1996).

In changing scenario, lack of methods necessary for identification of the species is a major hurdle for enforcement agencies. It is also difficult to identify and isolate the derivatives, used in various forms in trade under different trade names (Mukherjee 1996). Bone, tusk, antler and rhino horn are the major items in wildlife trade and are also used in various parts and product form. Characterization of species from parts and product of these items and its identification have yet not been undertaken for majority of the wildlife parts. Fake items are also common in trade, like musk and bear bile are often adulterated with bee-wax and jelly, the buffalo horn or stone are used to imitate rhino horn whereas the fur of dog, horse and calf are used to imitate tiger skin. To strengthen the law, scientific identification of the material used in offence cases were of foremost importance, therefore, this study is aimed to provide base line data using all possible and available morphometric, analytical and molecular techniques to identify major wildlife parts and products of bone, antler and horn reported widely in trade.

1.2. Objectives

1. Determine morphological, crystallographic (XRD & XRF) and DNA characteristics of major bones of tiger and leopard, and
2. Determine morphological, crystallographic (XRD & XRF) and DNA characteristics of rhino horn and antler of chital, sambar and swamp deer species.

1.3. Hypothesis

1. Morphological, crystallographic (XRD & XRF) and DNA characteristics of tiger and leopard bones are species specific, and
2. Morphological, crystallographic (XRD & XRF) and DNA characteristics of Rhino horn and antler of chital, sambar and swamp deer are species specific.

1.4. Species in the study

This study is aimed to characterize the animal products which are in high demand for their various uses. These products are bones of tiger (*Panthera tigris*) and

leopard (*Panthera pardus*), horn of greater Indian one horned rhinoceros (*Rhinoceros unicornis*), antlers of chital (*Axis axis*), sambar (*Cervus unicolor*) and swamp deer (*Cervus duvaucelî*). Other than these, antlers of two more species were also analyzed viz. hog deer (*Axis porcinus*) and barking deer (*Muntiacus muntajak*) for comparison. Ivory of Asian elephant (*Elephus maximus*) and African elephants (*Loxodonta africana*) were analyzed using similar techniques for comparison purpose.

1.5. State of knowledge

Morphometry has been used extensively to characterize species, but, very few morphometric studies have been conducted to characterize tiger and leopard bones (Pocock 1939; Pandit 1994), rhinoceros horn (Kingdon 1979; Dinerstein 2003) and antlers (Bubenik 1968; Martin 1975; Caro *et al.* 2003). Scanning electron microscopic technique has been used to study bones (Boyde and Jones 1972; Holden *et al.* 1995; Ubelaker *et al.* 2002) but it has not been used to study tiger and leopard bone surface morphology. There are few studies on scanning electron micrography of African rhinoceros horn (Lynch *et al.* 1973; Hsieh *et al.* 2001). Studies have been conducted on antler microscopy and histology by several workers (Speer 1983; Fenessy and Suttie 1985) but scanning electron microscopy has not been used.

X-ray diffraction of bones has been studied (Eanes 1965; Tannenbaum and Termine 1965; Tuross *et al.* 1989; Chipra and Bish 1991) but, again no study is available on X-ray diffraction for tiger and leopard bones. Similarly there is no study on antlers and rhino horn using X-ray diffraction. Studies on composition and elements of bones have been carried out (Batdemberel *et al.* 1999; Skinner 2000; Sangaa *et al.* 2002) but, there is no elemental analysis of tiger and leopard bones through X-ray fluorescence and inductively coupled plasma- mass spectrometry. Several authors (Lee-Thorp *et al.* 1992; Hall-Martin *et al.* 1993; Hart *et al.* 1994; Emslie *et al.* 2001; Amin *et al.* 2003) have used chemical techniques to differentiate species and geographical source of the African rhinoceros horn, based on the variation in the concentration of elements and isotopes and their ratios. Studies have been also conducted on antlers composition and elemental analysis (Chapman 1975; Sunwoo *et al.* 1995). Thermo gravimetric analysis technique commonly used to understand

changes in organic and inorganic states and has been used to explore its utility for tiger and leopard bones, antler and rhinoceros horn.

Protein profile of African rhinoceros horn has been studied by Block (1939) and Butler (1990). Few studies have been done on tiger bones (Wetton *et al.* 2004; Xu *et al.* 2005) using DNA analysis. Molecular studies available for African rhinoceros have used tissue, horn and blood, mainly for phylogenetics (Ashley *et al.* 1990; Swart *et al.* 1994; Tougard *et al.* 2001). Only few studies have been conducted to identify rhino horn using molecular techniques in Asia and Africa (Ali *et al.* 1999; Hoesch and Steven 2004; Hsieh *et al.* 2003). Similarly genetic determination of size of antler and shape has been studied (Suttie 1990) but, identification of antlers of different species through DNA has not been examined.

1.6. Collection of samples and literature

Reference materials needed for characterization were collected from Forest Departments of Uttarakhand, Uttar Pradesh, Madhya Pradesh, Assam, West Bengal, Karnataka, Kerala and Tamil Nadu of India. Samples were also collected from Wildlife Preservation Office, New Delhi, Wildlife Teaching and Forensic Laboratories of Wildlife Institute of India.

Reference literatures were collected from libraries of various Institutions viz. Wildlife Institute of India, Punjabi University, Patiyala, Central Forensic Science Laboratory, Kolkatta, Wadia Institute of Himalayan Geology, India Institute of Science, Bangalore and Jwaharlal Nehru University, Delhi. Other than these libraries internet was also used for referencing materials mainly through Science Direct.

1.7. Techniques used

Different techniques were used to characterize the above mentioned animal products so that even if it is transformed into powders the product may be identified using its characteristics. Application of different techniques was to help in

characterization of these animal products. In this study morphometry has been used to study the morphological features which can support the characterization of different animal products under observation. Each and every article has its unique characteristics. But, when such samples are processed the morphological features are destroyed which were present when the article was intact, thus in such circumstances some characteristics were required to identify it even when it is processed.

It is always better to provide baseline data determined by various analytical techniques for species characterization. This is because that if one technique fails or some how not able to distinguish one article from other, a combination of techniques would be of immense help in confirmation and increase the accuracy of the identification. Scanning electron microscopy is one of the techniques used to study the surface morphology of object. This technique was used to see if there were any differences in the surface topography of one article with other, so that it may be used for the samples which are available in pieces. X-ray diffraction was used to know the crystalline structure of these animal products and to differentiate it from similar products. X-ray fluorescence, an instrumental technique was used to know the qualitative elemental composition as quantitative analysis through this technique heavily depends on matrices and standards, which were not available for these items. Only mutual comparison of the XRF intensities of different elements was indicated for these animal products. Quantitative analysis were accomplished using Inductively coupled plasma – mass spectrometry which are less sensitive to matrices and multi-element salt standard could be used to determine these animal products quantitatively. Thermo gravimetric analysis technique was used to examine the nature of weight loss and the nature will remain more or less same for a particular article. Protein and DNA techniques were used in characterization these items to distinguish it from related products.

1.8. Utilization of various technical facilities

It was not possible to complete all analysis at the Wildlife Institute of India, Dehradun therefore, many other Institutions were helpful in accomplishment of objectives. Most of the analysis on morphometry and DNA were performed at the

Wildlife Institute of India, scanning electron microscopy, X-ray diffraction, X-ray fluorescence, Inductively coupled plasma – atomic emission spectrometry and mass spectrometry were completed at the Wadia Institute of Himalayan Geology, Dehradun, Thermo gravimetric analysis and strontium isotopes were analyzed at the Instrumentation Facility of Indian Institute of Technology, Roorkee and carbon and nitrogen isotopic analysis at the Indian Institute of Sciences, Bangalore and Gandhi Krishi Vikash Kendra, Bangalore.

1.9. Limitation of the study

The present study is constrained by the availability of wildlife products needed for study as these are highly restricted and required lots of permission to get authentic samples. Similarly all instrumental facilities required for this study were not available at our Institute therefore dependency was on the time slots provided by various other Institutes.

1.10. Organization of the thesis

Major aims of the thesis were to characterize bones of tiger and leopard, rhinoceros horn and antlers of different deer species using different techniques ranging from morphometric to analytical such as scanning electron microscopy (SEM), X-ray diffraction (XRD), X-ray fluorescence (XRF), inductively coupled plasma – mass spectrometry (ICP-MS), Thermo gravimetric analysis (TGA), Protein profile and DNA analysis. All working chapters are independent as these deals with different types of wildlife products. References of each chapter have been given at the end of each chapter separately for lucidity. The number of samples used for different methods in each chapter has been given in tabular form under respective method sections.

The total efforts for the thesis work and its outcome have been incorporated into four chapters. Chapter 1 "Characterization of wildlife products for identifying species to deal wildlife offence cases: a need for conservation", describes about the problem of wildlife trade in general, background to the research, highlight the lacunae in the current status of knowledge about these animal products, need to characterize

the animal products, requirement of various techniques for characterization, limitation of the study and organization of the thesis.

Chapter 2 "Characterization of tiger and leopard bones", discusses about characterization of major bones of tiger and leopards. It also deals with extent of tiger and leopard bone trade and literature review on characterization of bones. Materials and methods used viz. morphometry, scanning electron microscopy, X-ray diffraction, thermo gravimetric analysis, X-ray fluorescence, inductively coupled plasma – mass spectrometry and DNA analysis for characterization of bones of these species. This chapters also describes results of these analysis, discussion about the results, distinction between bones of both the species and literature used were cited at the end of this chapter.

Chapter 3 "Characterization of rhinoceros horn", deals with characterization of horn of greater one horned rhinoceros compared with buffalo horn, discusses about introduction to the species, status of their trade, cause of their trade, studies conducted on these species related to this thesis, materials and methods used viz. morphometry, scanning electron microscopy, X-ray diffraction, thermo gravimetric analysis, X-ray fluorescence, Inductively coupled plasma – mass spectrometry, protein profile and DNA analysis for characterization of these species. Results of the analysis, discussion about the distinction of the two types of horn have been described. Literatures referred in this chapter are cited at the end.

Chapter 4 "Characterization of antler", is to characterize and distinguish antlers of different deer specie. This chapter discusses about introduction to deer species in India, status and cause of their trade, literature review on antlers, materials and methods used viz. morphometry, scanning electron microscopy, X-ray diffraction, thermo gravimetric analysis, X-ray fluorescence, inductively coupled plasma – mass spectrometry, protein profile and DNA analysis for characterization of these species. Results of the analysis, discussion about the distinction feature among antlers of different species and literature that were referred are cited at the end of this chapter.

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CHAPTER 2

Characterization of tiger and leopard bones

2.1. Introduction

Poaching of tiger (*Panthera tigris*) and leopard (*Panthera pardus*) for their bones and skin is one of the major conservation threat for these species in India in addition to habitat fragmentation. A report of the Wildlife Protection Society of India revealed that in India, 95 tigers were poached in 1994, 123 tigers in 1995, 52 tigers in 1996, 89 tigers in 1997 and 36 tigers in 1998 (http://www.nbs.it/ tiger/Tiger2.html). Similarly seizure of more than 45 kg of tiger bone in 1997, 65 kg in 1998 and 312.5 kg in 1999 (http:// www.nbs.it.tiger/Tiger7.html) also gives an indication of persistent trade of tiger bones. Bones of tiger and leopard are in illegal trade, mostly due to their use in oriental medicines (Mills and Jackson 1994). The tiger bone trade is spread globally in commercial market, as it is considered more valuable than its skin (Jackson 1993). Virtually every part of the tiger is used in Chinese medicine (Mills and Jackson 1994). Tiger bone is most commonly used to treat rheumatism, weakness and stiffness or paralysis.

Though these species have been placed in Appendix I of CITES and Schedule I of Wildlife (Protection) Act, 1972 to provide highest protection (Nowell 2000). In absence of proper methods for characterization of their parts, enforcement agencies faces problem in conviction of the offender. Therefore, characterization of bones of these species is very important. Thus, present thesis aimed to characterize major bones of tiger and leopard through various methods.

Morphometric technique is extensively used to differentiate bones of various species. Diersing and Hoffmeister (1977) described the method of taking skull measurement for shrews. Different methods have been used to measure bones of small mammals by various workers (Merriam 1889; Anthony 1925; Morrison – Scott 1939;

Hoffmeister and Mohr 1957; Hall 1962; Setzer 1963; Corbet 1964). Pocock (1939) described the skull characteristics of Primates and Carnivores. Various workers (Kirsch and Poole 1972; Poole 1975 and 1977) have used skull morphometry to determine the distribution of two species of grey kangaroo. Measurements of bones mainly skull has been used to establish phylogenetic relationship. Skinner (1942) had referred to skull and postcranial elements of *Eqqus conversidens.* Mook (1921) has worked on skull of crocodilia and derived affinity between them. Kelson (1946) reported the differences in the bones of Bobcat (*Lynx*) and House cat (*Felis*). Pandit (1994) has reported the osteology of Indian Tiger (*Panthera tigris tigris*) in detail. Taluja *et al.* (2001) took the osteometric measurements of the scapula and cervicle vertebrae of tiger (Taluja *et al.* 2002). Singh (1997) has described the tiger skull and gave few skull measurements.

Pocock (1939) noticed differences in the skull of the Asiatic lion and African lion. He reported that Asiatic lion has a more strongly developed sagittal crest than the African lion and the infraorbital foramen in Asiatic lion is divided in to two orifices, whereas it is undivided in the African lion. Todd (1965) compared the skull of Asiatic lion with African lion and reported differences into facial and cranial portion between two populations. He noticed that the skull of Gir lion tends to be broader but shorter in the facial region than the African lion, while in cranial region he found a reversed tendency. Ganatra *et al.* (1990) tried to estimate the age of Asiatic lion through skull bones measurements. Ray *et al.* (1994) studied the skeleton of fore arm of leopard (*Panthera pardus*). Archana *et al.* (1998) studied the skull bones of Yak (*Bos grunniens*) in north western Himalayan region of India. Sarma *et al.* (2002) noticed only one large mental foramen in the mandible of leopard cat (*Felis bengalansis*) whereas dog and domestic cat usually have two mental foramina. They also found comparatively smaller ramus unlike in dog and cat and extensive masseteric fossa and the coronoid process.

Pocock (1939) reported that the skull of tiger and lion is difficult to distinguish. He further reported that on the average tiger's skull is shorter in its facial and longer in its cranial portions. The Indian tiger skulls vary considerably in length and zygomatic width.

Apart from the skull size, the most noticeable variation is in the development of the sagittal crest. Pocock (1939) compared the skull of tiger and leopard and reported that the skull of leopard differs from a tiger's by being lower and flatter in the frontal region, the postorbital region is shorter so that the facial portion looks longer and more massive as compared with the cranial. The anterior nares are wider and the nasal bones shorter and carnassial teeth are smaller. The lower edge of the leopards mandible are slightly convex in the middle, so that the skull "rocks" slightly when placed on a flat surface, on contrary the mandible in tiger is slightly concave below, so that the skull never "rocks" when resting on a flat surface. Pocock (1939) found difference between tiger and leopard skull. Elledge *et al.* (2006) documented morphological differences in captive and domestic animals from their wild counterparts. They compared changes in both hard and soft tissues in captive and domestic animals with those in their wild counterparts including skull shape differences, brain size reduction, postcranial adaptations and digestive tract changes.

Scanning electron microscopic technique is to observe surface topography and it helps in differentiating surface structure of one type of sample from other. This technique is used mostly in geology, petroleum science and to study fossils. Now a day, it is being used to identify biological samples also. Ubelaker *et al.* (2002) used Scanning electron microscopy/ energy dispersive X-ray spectroscopy to distinguish dental and osseous tissue. They found that the proportions and amounts of calcium and phosphorous are important in distinguishing bone and tooth from other materials. However they could not differentiate between ivory, mineral apatite and some corals. Holden *et al.* (1995) studied effects of heat on bones and quantified the changes in ultra structure of the bone tissue. These changes were not profound at low magnification but, ultra structural features were specific to the associated temperature transitions and age of the deceased at higher magnifications. This study can be of immense use in case of burnt bones. The ultra structure of normal and pathological bones was well documented by Boyde and Jones (1972).

It is well known that the bones are composed of mineral apatite but a few studies have been conducted to characterize these materials. Chipra and Bish (1991) compared the bone of Seismosaurus with contemporary elk bone using X-ray diffraction technique. They reported that the crystallite size of Seismosaurs bone was found to be larger with less strain then the later. Studies conducted on pig enamel have suggested that bone apatite has a high affinity for fluorine (Eanes 1965; Tannenbaum and Termine 1965). Chemical constituents of bones can also be used as species specific characteristics if difference is consistently found between concentrations of elements among two species. Initial work by Tuross *et al.* (1989) on XRD & XRF for mineralized vertebrate tissues indicates differences in composition. They documented the progressive changes in both organic and inorganic components in wildbeest bone sampled of two longitudinal regions.

Bone is a composite material of collagen and slightly impure hydroxyapatite. The basic hydroxyapatite ($Ca_5 (PO_4)_3OH$) structure is hexagonal with space ground P63/m, while the values of their structural parameters often depends on the chemical thermal processing. Sangaa *et al.* (2002) studied on ground squirrel's bone detected all diffraction peaks within the hexagonal hydroxyapatite ($Ca_5 (PO_4)_3OH$) (sp.gr. $P6_3/m$) and cubic calcium oxide CaO (sp.gr.Fd3m) structures. Batdemberel *et al.* (1999) did comparative studies on crystal structure of fossil and recent bones. Bioapatite is a highly accommodative minerals and is able to store and release calcium and phosphorous, and several other ions such as sodium, potassium, magnesium, fluorine, carbonate and hydroxide and thus bone serves as mineral reservoir for the metabolic activities (Skinner 2000).

Villanueva *et al.* (1976) has studied the dating of bone using the application of differential thermal analysis and thermo gravimetric analysis and could successfully differentiate recent and old bones.

It is difficult to differentiate the tiger parts at individual level using morphological characteristics as it does not possess the distinctive characteristics of the individual (Xu *et al.* 2005). The studies of DNA provide great potential for research in anthropology, archaeology, forensic science, biology, and the evolutionary sciences (Herrmann and Hummel, 1994). There are, however, factors that limit the extent to which ancient DNA can be analyzed. The advent of the polymerase chain reaction (PCR) (Saiki *et al.*, 1985) has made it possible to amplify and analyze DNA isolated from ancient bones, mummified tissue, and even fossilized material. Polymerase chain reaction (PCR)-based DNA typing methods combined with standard anthropological approaches now play an important role in bone identification (Cattaneo *et al.* 1999; Imaizumi *et al.* 2002). Yanga *et al.* 2004 reported on the development and application of methods for using DNA analysis for species identification of archaeological salmon bone from the site of Namu on the central coast of British Columbia. Short fragments (less than 300 bp) of mitochondrial DNA from the control region (D-loop) and cytochrome b gene were targeted for amplification using the polymerase chain reaction (PCR). Four species: coho, sockeye, pink and chum salmon were identified from the samples. The results demonstrate the applicability of the ancient DNA technique to species identification of even single salmon vertebrae from archaeological sites in the Pacific Northwest of North America. In investigating criminal cases of poaching and smuggling involving tigers (*Panthera tigris*), the number of tiger individuals involved is critical for determining the penalty (Xu *et al.* 2005).

Wetton *et al.* (2004) tested species-specific PCR for the presence of tiger bone DNA in traditional Chinese medicine. They developed a faster, simpler and cheaper test for screening large quantities of TCMs in a single tube assay in which rapid thermo cycling is combined with the simultaneous detection of a fluorescent double stranded DNA binding dye to detect the presence of DNA fragments matching tiger-specific primers without recourse to post-PCR processing.

2.2. Objective

Determine morphological, crystallographic (XRD & XRF) and DNA characteristics of major bones of tiger and leopard.

2.3. Hypothesis

Morphological, crystallographic (XRD & XRF) and DNA characteristics of tiger and leopard bones are species specific.

2.4. Materials and methods

Bones used for the study were collected from Forest Departments and Forensic laboratory, Wildlife Institute of India. Characterization of bones of tiger and leopard were undertaken using morphometry, crystallography and DNA sequences. Table 2.1 indicates the number of samples analyzed for morphometry and analytical techniques.

Morphometric measurements are used to characterize major bones of tiger and leopards. The major bones included are skull, scapula, humerus, radius ulna, pelvic, femur and tibia fibula of both the species. Limited numbers of samples were tried as procuring authorized bone samples were difficult and tedious. Elemental analysis of tiger and leopard were determined using X-ray fluorescence and inductively coupled plasma – mass spectrometry. A sample each of tiger and leopard bone was examined using X-ray fluorescence for sixteen elements for screening. Further quantification of elements was tried using ICP- MS to note the differences among elements between the bones of these two species.

Table 2.1. Number of samples analyzed for morphometric measurements and different analytical techniques.

Techniques	Number of sample	
	Tiger	Leopard
Morphometry		
Skull	10	5
Scapula	10	11
Pelvic	8	2
Femur	13	8
Humerus	9	5
Analytical		
SEM	3	3
XRD	5	5
XRF	1	1
ICP-MS	3	2
TGA	3	1
DNA	2	2

2.4.1. Morphometric characteristics of bone

Bones used for measurements were cleaned using standard methods of Chapman and Chapman (1969). First these bones were boiled for three to four hours in NaOH or KOH to remove muscles and bones were bleached in 5% hydrogen peroxide for at least two hours. Using this method the materials were boiled in 10% NaHCO3 for four hours and left to macerate in the water added 10% NaHCO3 and 10% granule soap for five days. Cleaned bones were oven dried or sun dried.

Major bones like skull, scapula, humerus, pelvic and femur of tiger and leopard were used for measurements (Table 2.1). Various standard measurements suggested for bone measurements (Fig. 2.1 to 2.4) were used by Von Driesch (1976) and were recorded on datasheet. The measurement variables are provided in Table 2.2 to 2.7. Most of the measurements were taken in mm using vernier calipers, thread and measuring scale as per Miller *et al.* (1964) and Archana *et al.* (1998). Discriminate Function Analysis (DFA) (Blackith and Reyment, 1971; Davies, 1971) was used for identifying characters suitable for species identification. To assess the usefulness of univariate variable as discriminators

between the two species, discriminant coefficients and their corresponding probabilities of misidentification (Lubischew 1962) was calculated using SPSS program.

Table 2.2. Variables used in skull measurements.

Aspect	Variables
Dorsal	Maximum zygomatic width
	Facial length
	Post orbital constriction
	Maximum cranial width(across parital)
	Distance between temporal ridges at coronal sutures
	width of frontal shield
	Inter orbital width
	Width of nasal bones
	Maximum internal width of nasal aperture(oblique measurement)
	Maximum internal width of nasal aperture
	Nasal volume
Lateral	Total skull length
	Cranial height
	Length of sagittal crest
	Skull height
	Orbital height
	Orbital width
	Vertical distance from posterior alveolus P4 to orbit
	Height of tympanic bulla
	Height C1(from alveolus)
	Crown lengthC1
	Canine nasal distance(CND)
	Nasal length(NL)
	Orbital depth
	Orbital area
	Depth of jugal
Ventral	Skull base length
	Palate length
	Minimum distance from internal nares to anterior palatine notch
	Maximum length of tympanic bulla
	Width of tympanic bulla
	Zygomatic width at M1
	Maximum width between C1 alveoli
	Palate width between M1 alveoli
	Porion to porion
	Length of cheek teeth row
	Maximum crown length P4
	Maximum crown width P4

Table 2.3. Variables used for measurements of scapula.

Selected variables
Height (HS)
Diagonal height (DHA)
Greatest dorsal length (Ld)
Smallest length of the neck (SLC)
Length of glenoid cavity (LG)
Breadth of glenoid cavity (BG)
Circumference of glenoid cavity (CGC)
Length of coracoid process (GLP)
Width of scapula (maximum) (WS)
Length (Greatest) of scapula spine (LSS)
Height of scapula spine (HSS)
Width of supraspinous fossa (maximum) (WSf)
Width of infraorbital fossa (maximum) (WIF)
Beak –like projection near coracoid process

Table 2. 4. Variables used for measurements of pelvic.

Selected variables
Greatest length of one half (GL)
Length of the acetabulum on the rim (LA)
Length of the symphysis (LS)
Smallest height of the shaft of the ilium (SH)
Smallest width of the shaft of the ilium (SB)
Smallest circumference of the shaft of ilium (SC)
Inner length of the obturator foramen (IFO)
Greatest width across the tuber coxae (GBTc)
Greatest width across the tuber ischiae (GBTi)
Smallest width across the bodies of the ischiae (SBi)
Circumference of acetabulum (Ca)
Acetabulum tuber coxea distance (ATCD)

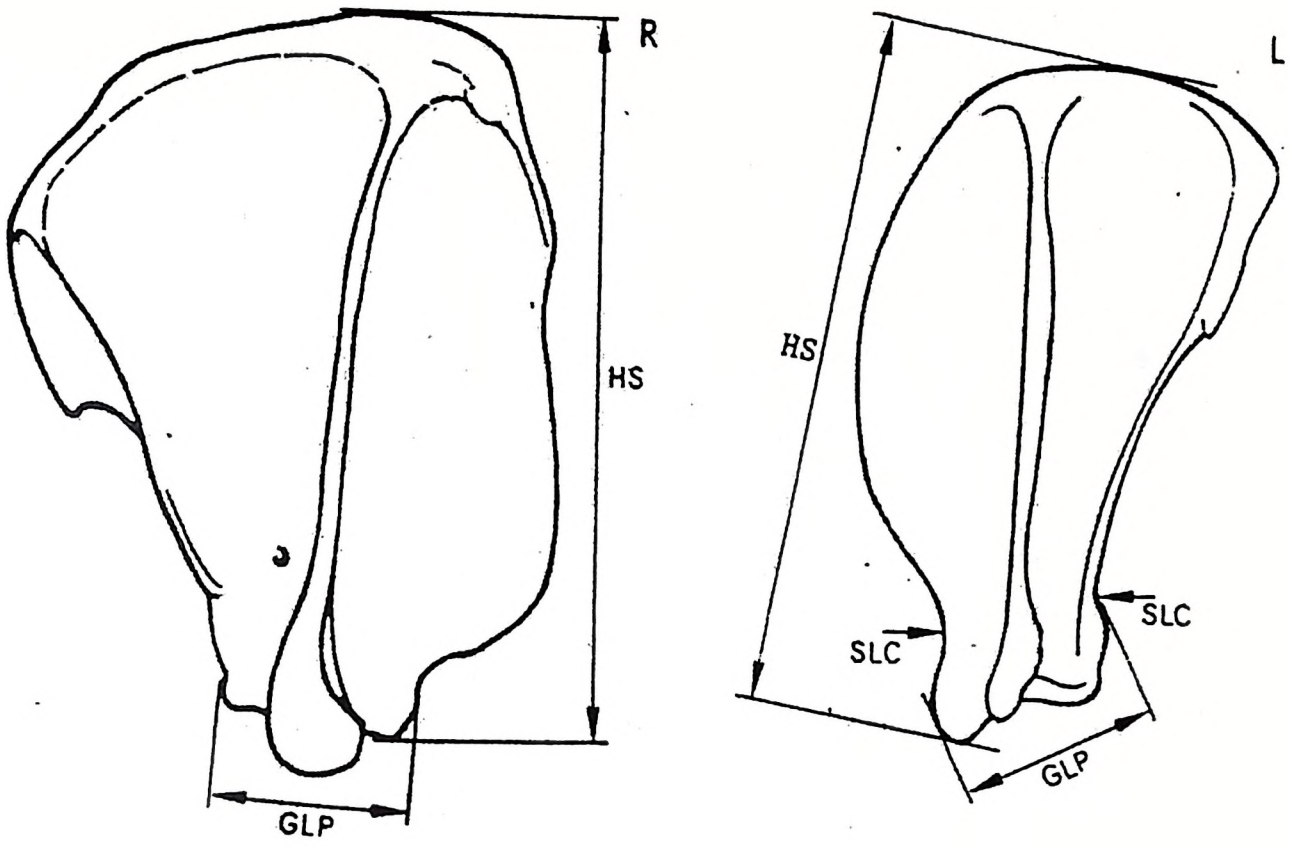
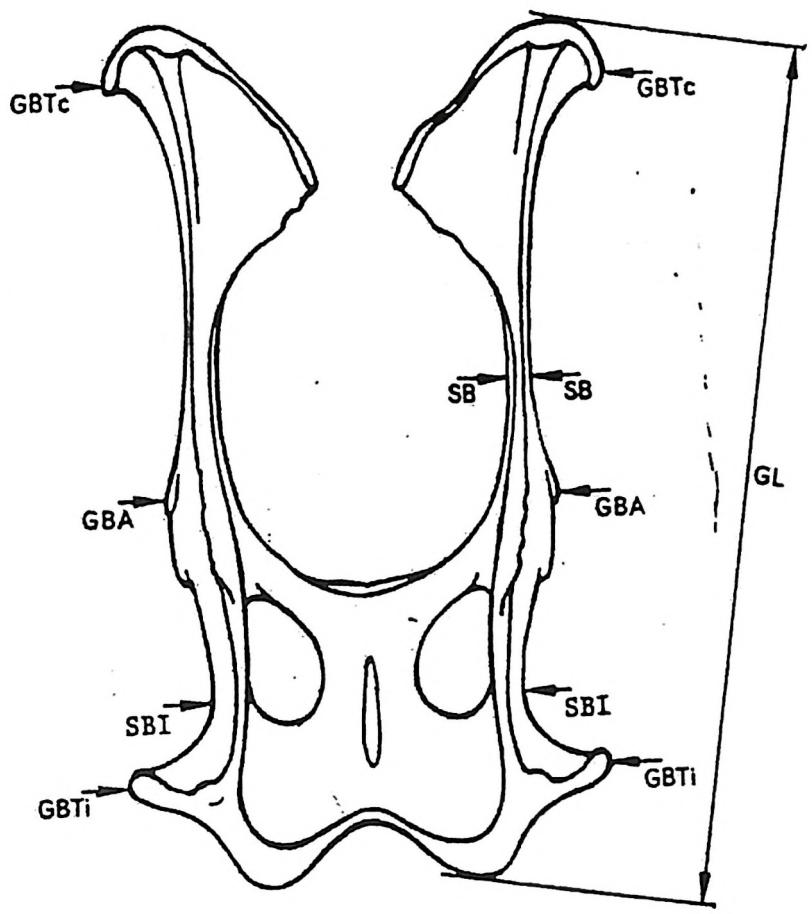


Figure 2.1. Variables used for scapula measurements (source Drisch 1976).

पुस्तकालय/Library
भारतीय वन्य जीव संस्थान, देहरादून
Wildlife Institute of India, Dehradun
वर्गीकरण सं. WII-8556
ACC NO
प्राप्ति दिनांक 17-6-2016
DATE OF RECEIPT
मूल्य/Price
प्रदायक/Supplier
हस्ताक्षर/Signature



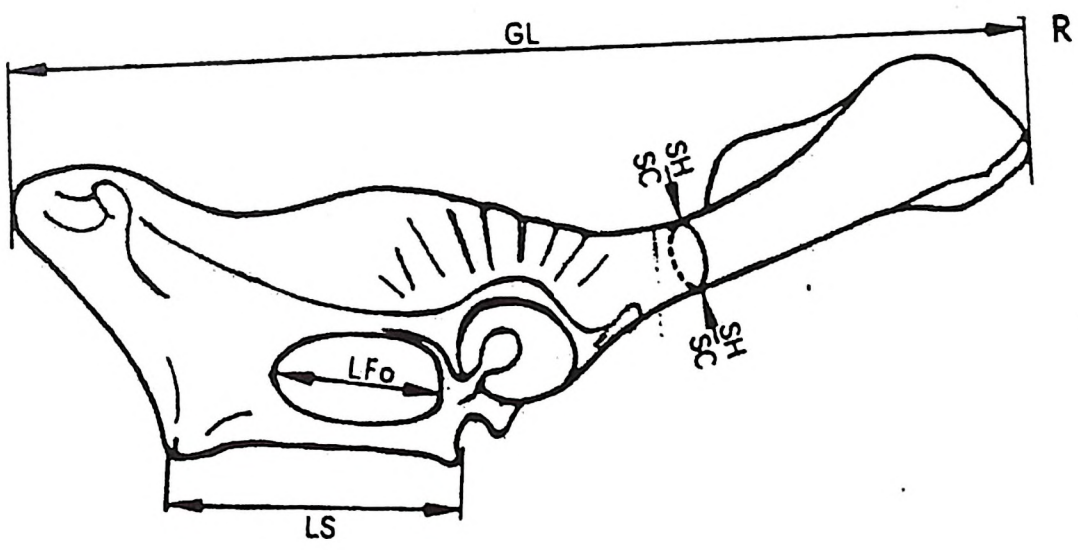


Figure 2.2. Variables used for pelvic measurements.

Table 2.5. Selected variables for measurement of humerus characteristics.

Variables
Greatest length(GL)
Greatest length of lateral part(GL1)
Greatest length from head(GLc)
Greatest width of proximal end (Wp)
Depth of proximal end(Dp)
Smallest width of diaphysis (SW)
Width of distal end(Wd)
Width of the trochlea (WT)
Inter condyler distance (Dd)
Distance from proximal end of the foramen (Dfpe)

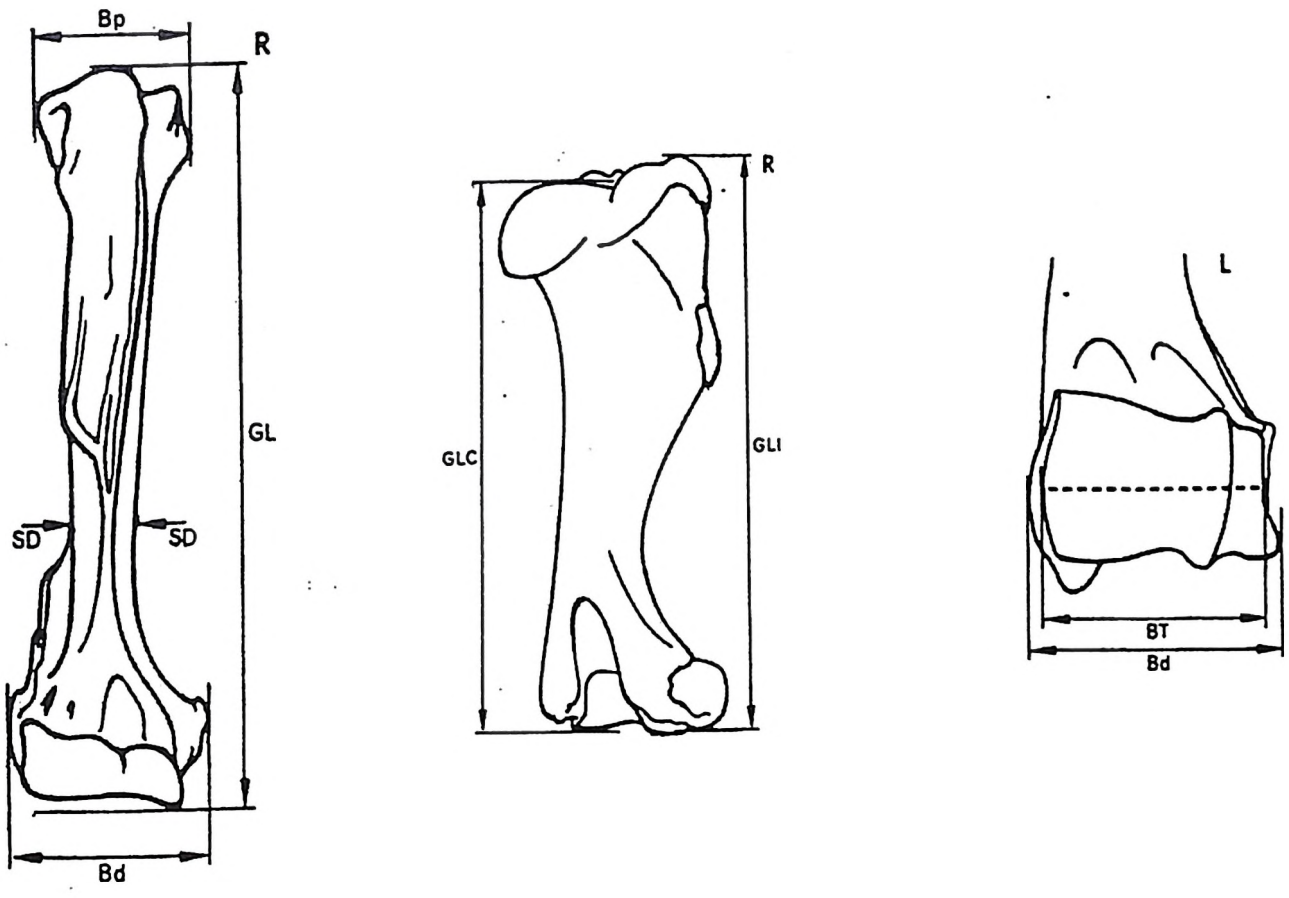


Figure 2.3. Measurement of selected variables of humerus.

Table 2.6. Selected variables used for measurements of femur.

Selected variables
Greatest length (Gl)
Greatest width of proximal end (Bp)
Greatest depth of the femoral head (Dc)
Smallest width of diaphysis (Sd)
Smallest circumference of diaphysis (Scd)
Greatest width of the distal end (Bd)
Inter condyler distance (Icd)
Depth of femoral head (Dc)
Greatest distance between trochelea (BTr)

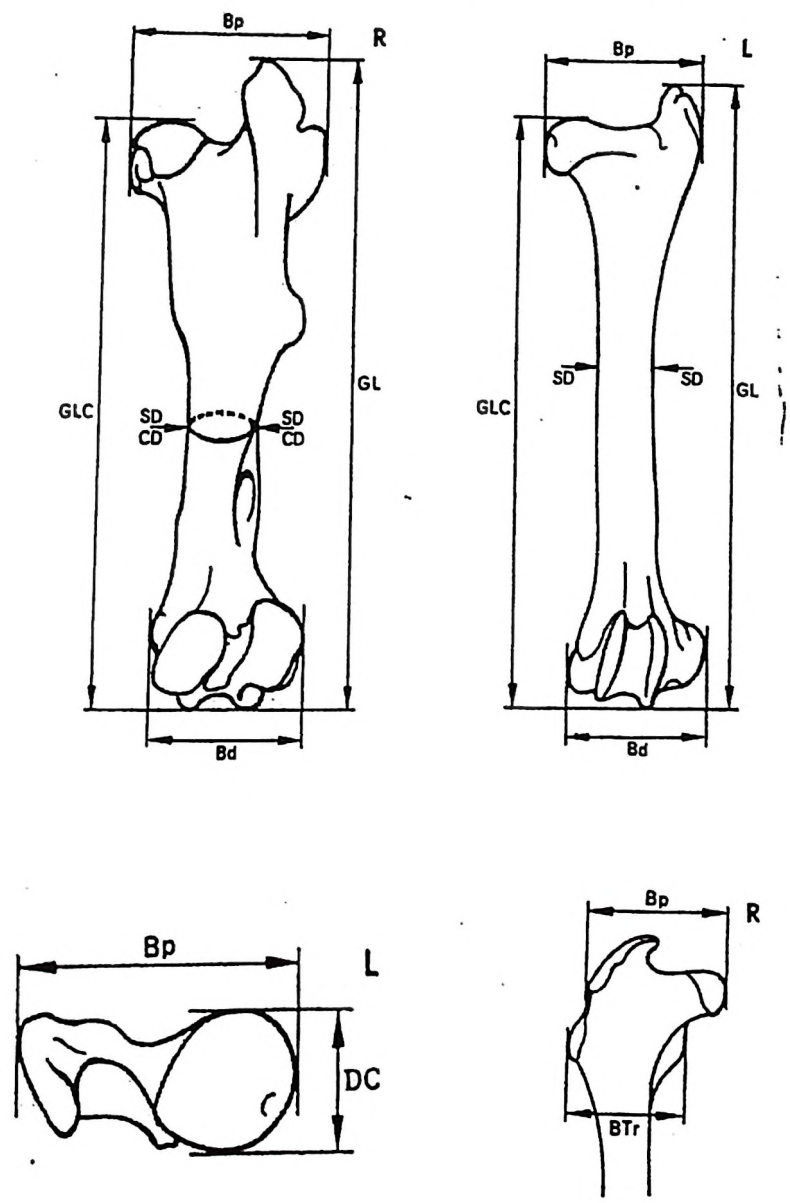


Figure 2.4. Variables used for femur measurements.

2.4.2. Scanning electron microscopy

Three tiger and two leopard femur bones were examined under the scanning electron microscope at three different sections viz. outer, cortex and core at different magnifications. Samples were prepared by cutting pieces of 5mm width, 5 mm length and 2 mm thickness, and cleaned properly using alcohol. These small pieces were mounted on the aluminum stub, using silver paste and left for drying and were sputter coated with gold (10-20 A^0) at 25 mA, 2.5 kV in argon atmosphere at 10-8 mbar/pa for 3 minutes using cool sputter coater (E5100 Series II, Polaron Equipment Ltd., U.K.) for better conductivity (Goldstein *et al.* 1981) using Scanning Microscope (PSEM 515, Philips, Holland). Micrographs were captured digitally using software Digital Image Scanning System (DISS 5, Point Electronics, Germany). Micrographs were compared at all portions, to note the species specific characteristics.

2.4.3. X-ray diffraction

Five samples each of tiger and leopard bones were ground uniformly for 15 minutes (200-300 mesh size) under ball mill. 0.3 to 0.4 g of powdered sample used for analysis was filled evenly in a sample holder and placed under the diffractometer for analysis. X-Ray Diffractometer (Pan Analytical, Philips, Holland) was used at 55 mA current, 40 kV voltage, Angle (2θ) - 25^0 to 55^0. Samples were tightly and homogenously loaded on a glass slide or in a sample holder for analyzing diffraction pattern. The software, X' Pert Pro was used for output of diffractogram.

2.4.4. Thermo gravimetric analysis (TGA)

In thermo gravimetric analysis, weight loss is noted with increase in constant temperature. This was carried out for tiger bones (n=3) as well as leopard bone (n=1) at Indian Institute of Technology, Roorkee. Approximately 10 mg of samples were burnt till 1400°C under nitrogen medium 100 ml/min along with the reference alumina in the thermal analyzer, Perkin Elmer, USA. Pyris Diamond software was used to print the thermographs.

2.4.5. Elemental analysis

2.4.5.1 X-ray fluorescence

X-ray fluorescence was used to know the screening of the bones for elements. A sample of each tiger and leopard bone was used for X-ray Fluorescence analysis. Samples were ground and pellets of less than 10 mm thickness were prepared using two-three drops of lithium borate or poly vinyl alcohol for binding the pellet and it was pressed under pressure pump and left for five minutes until pellet was formed. Samples were desiccated overnight and analyzed under X-ray spectrometer (Siemens SRS 3000, Varian, Australia). Three types of analyzing crystals were used to analyze namely, LiF 200, Ge and OVO55.

2.4.5.2 Inductively coupled plasma – mass spectrometry (ICP-MS)

For ICP-MS, teflon crucibles were cleaned, labeled for various samples. Approximately 0.1 grams of powder sample was measured and 10-15 ml of mixture of concentrated nitric acid and hydrofluoric acid in 1:2 ratios was added to it. Sample was warmed, till the sample dried. Again the same acid mixture was added and the sample was warmed. Then, five ml. of perchloric acid was added to this sample and boiled without lid. Same step was followed and completely dried sample was extracted with 20% hydrochloric acid. The sample solution was made-up to 200 ml by adding distilled water and kept ready for instrumental analysis using Perkin-Elmer SCIEX ICP-Mass Spectrometer model ELAN DRC-e, USA. Instrumental concentrations of various elements were transformed into parts per million using standard formula.

2.4.6. DNA analysis

Scraping gave the best result among drilling, cutting and grinding and 200 -400 mg samples were scraped. The quality of the DNA extracted from long bones is better than extracted from skulls or ribs protocols (Matsuda 2005). Therefore, two femur bones each of tiger and leopard were used for extraction of DNA. Two protocols used to extract

DNA, were of DNeasy Tissue Kit (QIAGEN, Germany) for animal tissue with slight modification of adding 0.39 mM DTT along with 180 µl ATL and Biorobotic methods using EZ1 Forensic card, (Qiagen Instruments, AG, CH-8634, Hambrechtikon). Decalcification was performed in both the method using 0.5 M ethylene diamine tetraacetic acid (EDTA, Life Technology Co. Ltd, Grand Island, NY, USA) at 56 ^{0}C overnight. Our preliminary work on DNA extraction indicates that, in Biorobotic method, 40 mg of powdered sample was used. 6-10 µl extracted DNA was resolved on 0.8% gel, and photographed (UVP Bio-imaging System, Cambridge, U.K.) under UV light. Further, the DNA fragments was cut and subjected to cleaning following the protocol of Qiagen gel purification kit, Germany.

Cytochrome b and 16S rRNA genes are located in the mitochondrial genome and highly conserved nucleotide sequences are known to be species-specific. These genes have been used for species determination in phylogenetic and forensic investigations (Kocher *et al.* 1989; Hsieh *et al.* 2001; Wan and Fang 2003). Therefore, following partial fragments of cytochrome b gene of 381 bp (Kocher *et al.* 1989) and 16S rRNA gene of 550bp (Mitchell *et al.* 1993) of mitochondrial DNA were chosen for species identification.

Primers used for cytochrome 'b' gene:

Forward, 5'- CCATCCAACATCTCAGCATGATGAAA-3'
Reverse, 5'-GCCCCTCAGAATGATATTTGTCCTCA-3',

Primer used for 16S rRNA gene:

Forward, 5'-CGCCTGTTTATCAAAAACAT-3'
Reverse, 5'-CTCCGGTTTGAACTCAGATC-3'

PCR amplification reaction was carried out in a volume of 50 µl containing 1x-PCR Buffer (50mM KCl, 10mM Tris HCl (pH 8.8), 0.08% Nomidet P40),1.5mM $MgCl_2$, 0.5 mM dNTPs, 0.2 µM of each primer, 0.1mg/ml BSA, 1U Taq polymerase, and 50-100ηg of DNA template. The reaction mixture was initially heat denatured at 94^{0}C for 2 min followed by 30 cycles of denaturation at 94^{0}C for 45 sec., annealing at 55^{0}C for both

primers, extension at 72 ^{0}C for 45 sec, with a final extension step at 72^{0}C for 10 min. Amplified PCR products were purified using QIAquick® PCR Purification Kit (QIAGEN, Germany). 6 µl of amplification products were resolved on a 2% agarose gel in 1xTAE buffer, stained with ethidium bromide and photographed under UV. For sequencing, PCR was set using 2 µl RR big dye, 1 µl 5X buffer, and PCR template. Amplified PCR products were purified with ethanol precipitation and the DNA sequences were observed on the automated DNA sequencer ABI 3130 (Applied Bioscience, USA).

Further DNA sequences of cytochrome b and 16S rRNA genes of *Panthera tigris* and *Panthera pardus* from NCBI data base were aligned using, Bioedit version 5 (Hall 1999) to see the pattern of variability in data. Nucleotide polymorphism for the genes was noted. The restriction enzyme digestion map was identified by software, Webcutter 2.0 for the cytochrome b and 16S rRNA genes restriction sites specific to tiger or leopard were identified. Then, Universal primer suitable for both species was developed using software, Primer3 version 4 for dealing smaller fragments of DNA.

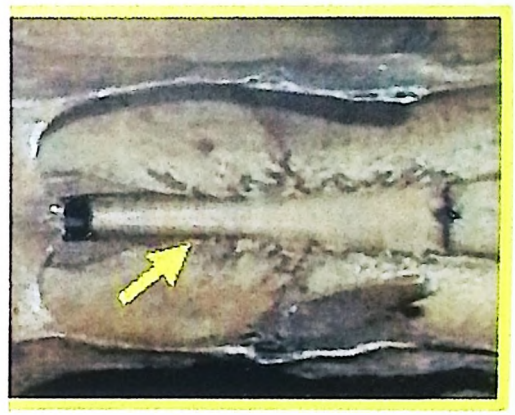
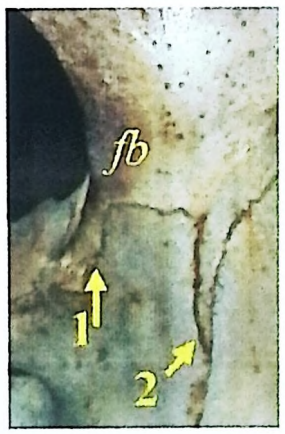
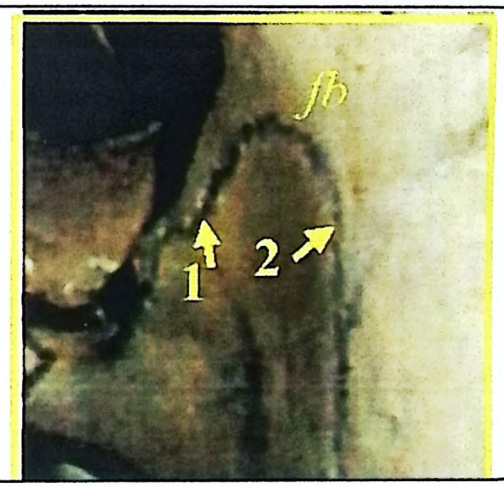
2.5. Results

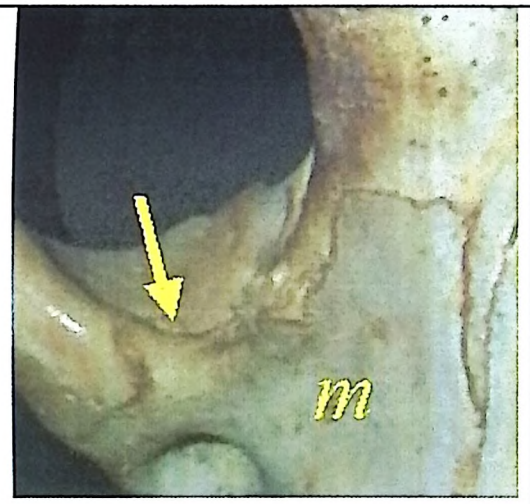
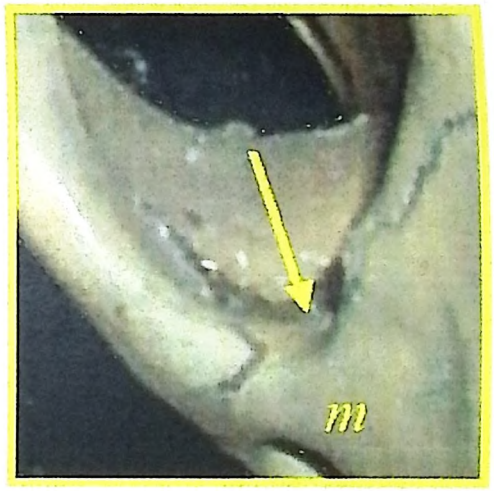
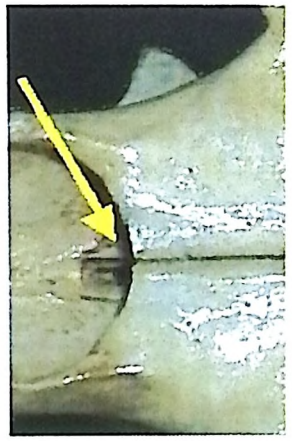
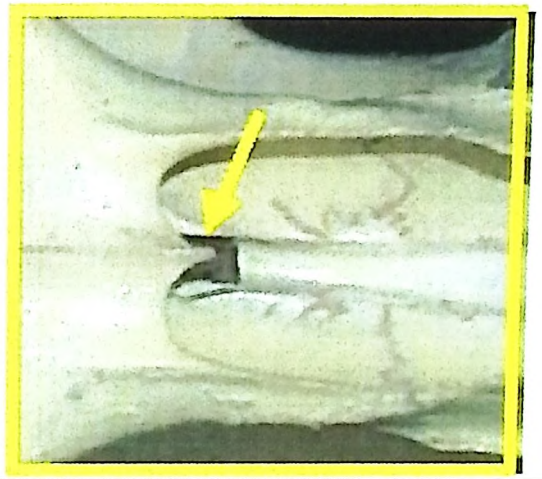
2.5.1. Morphometry- a basis for identifying major tiger and leopard bones

2.5.1.1. Morphological features

Table 2.7 indicates major differences between skull of tiger and leopard. These differences can be used to differentiate skull of tiger from leopard. Differences between scapula of leopard and tiger are given in Table 2.8. Differences in other bones were not studied but these were tried to differentiate based on morphometric measurements.

Table 2.7. Differences between tiger and leopard skulls.

Variables	Tiger	Leopard
1. Shape of presphenoid body	Short and wide, widened medially 	Long and narrow, with no median widening 
2. Length difference between orbital part (1) and nasal part (2) of the frontal bone (*fb*)	Orbital part is smaller than nasal part 	Orbital part is longer than nasal part 

3. *Extend of maxilla in relation to orbital cavity*	Maxilla (m) extend up to posterior margin become a part of orbital cavity 	Maxilla do not extend posteriorly, hence do not become a part of orbital cavity 
4. Posterior nasal spine	Absent 	Present 

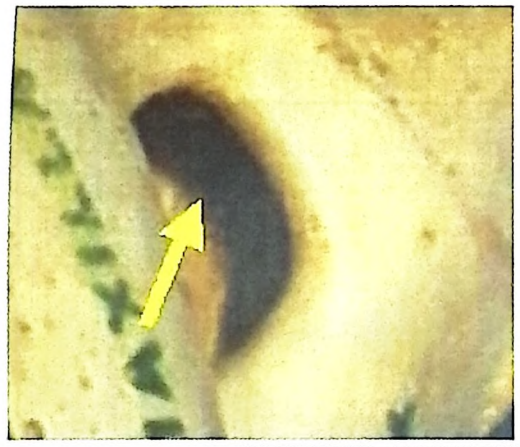
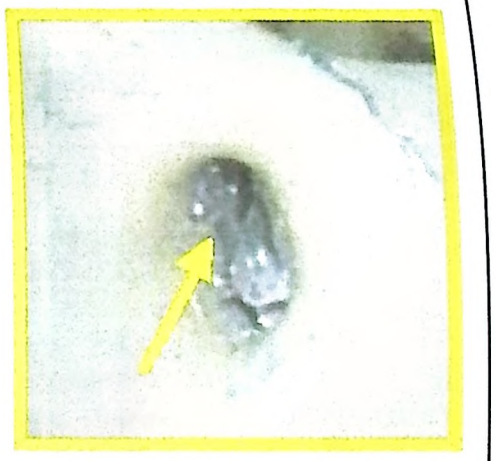
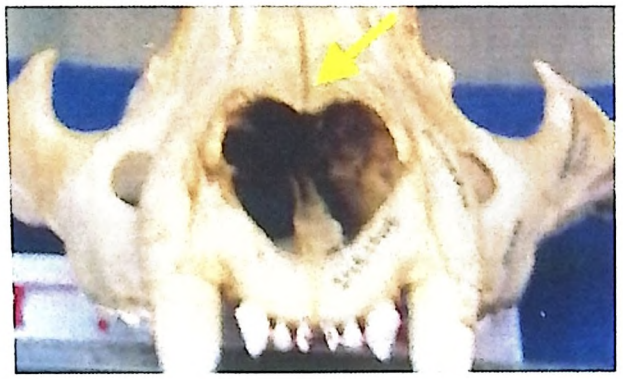
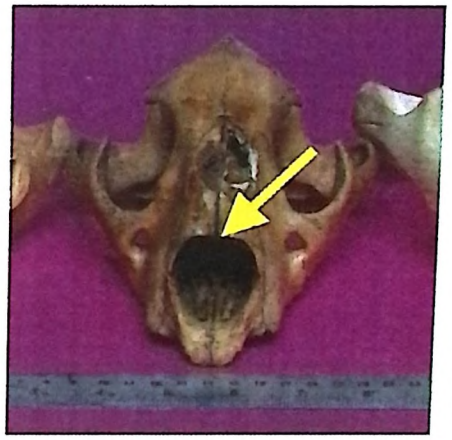
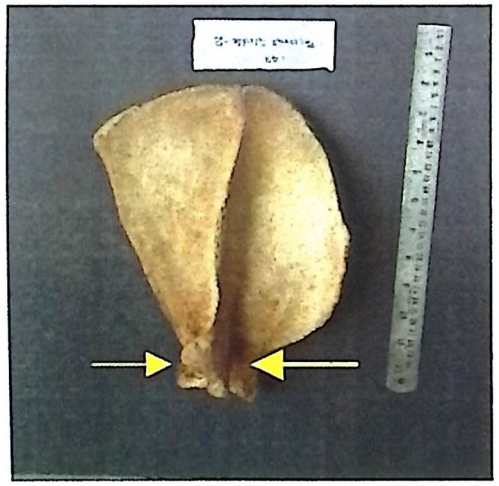
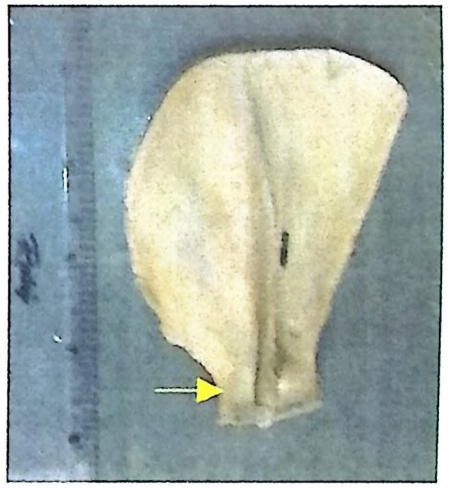
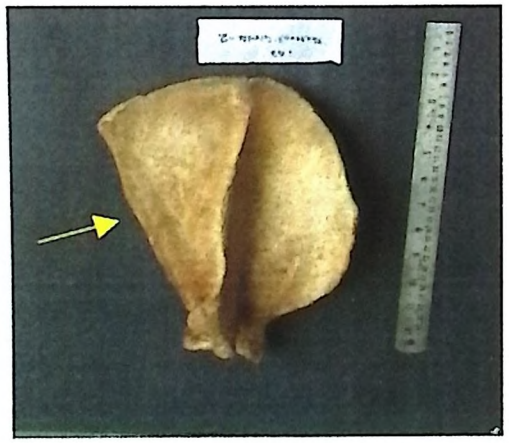
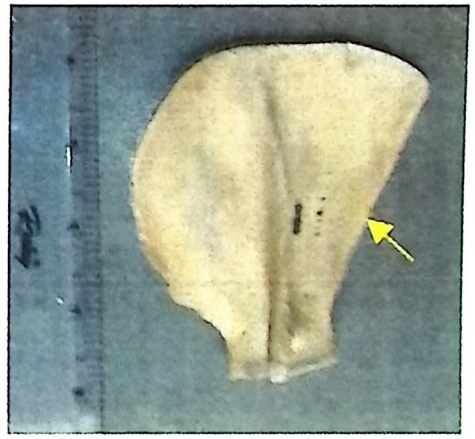
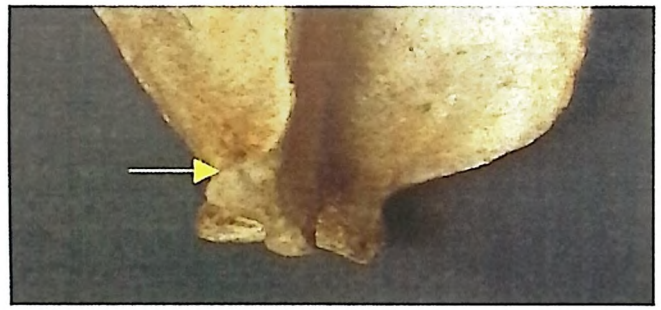
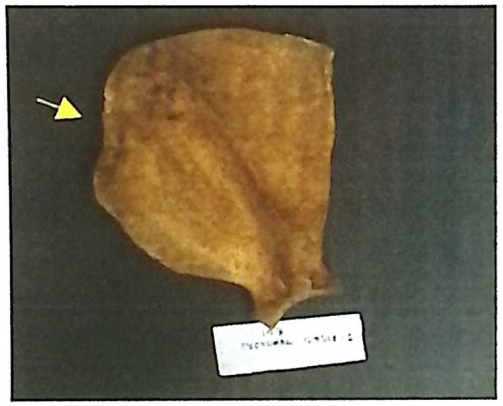
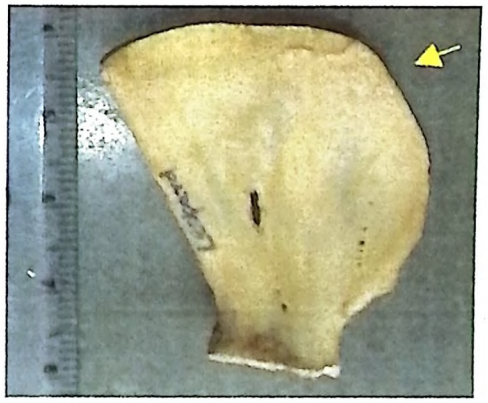
5. Shape of the infraorbital foramen	Large and oval 	Small and round 
6. Upper edge of vomer	Forms two curves and is 'M' shaped 	Forms one curve and is 'C' shaped 

Table 2.8. Differences in scapula between tiger and leopard.

Variables	Tiger	Leopard
1. Neck	Neck is narrow 	Neck is wide 
2. Infraspinous edge	Less slanting 	More slant 

Variables	Tiger	Leopard
3. Metacromion edge	Curved edge 	Triangular edge 
4. Supraspinous edge	Depression in the curve 	Depression not noticed, the edge has smooth curve 

2.5.1.2. Morphometric measurements

Various defined measurements of major bones viz. skull, scapula, pelvic, femur and humerus of tiger and leopard were taken according to Driech (1976). The Cranial measurements of tiger (n=10) and leopard (n=5) were analyzed. Of the 38 variables used (Table 2.2), five variables viz. maximum zygomatic width (MZW), facial length (FL), total skull length (TSL), crown length of canine (CL C1) and nasal length (NL) played major role in differentiating tiger skull from leopard.

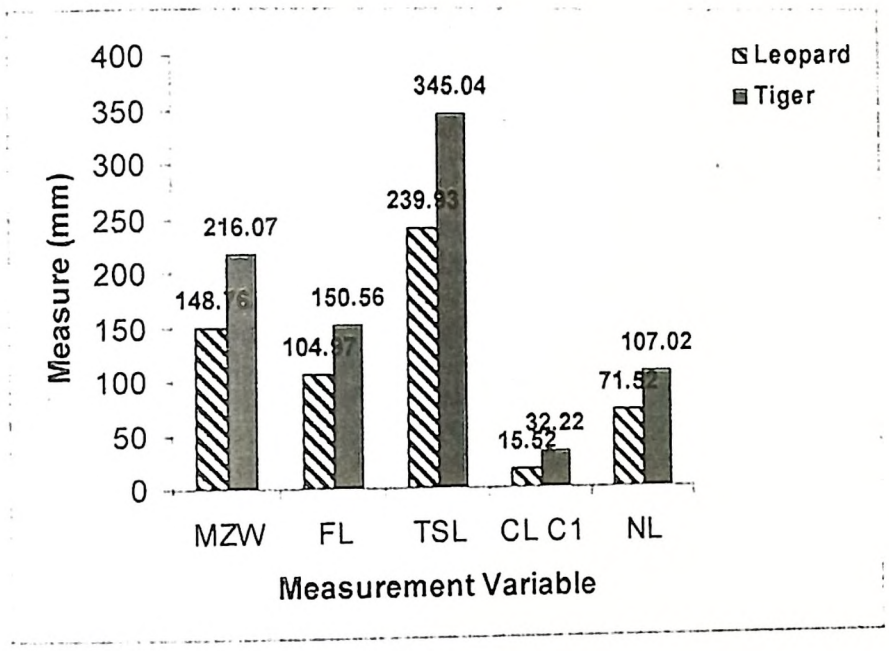
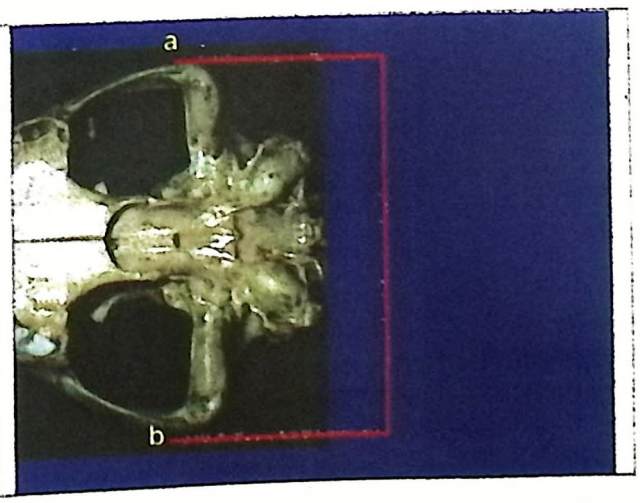
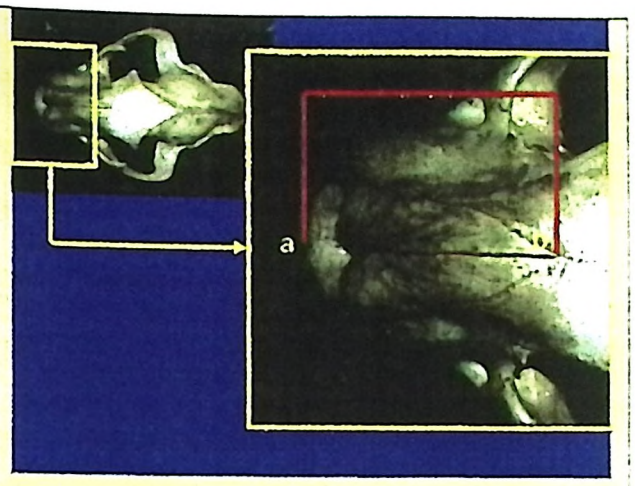
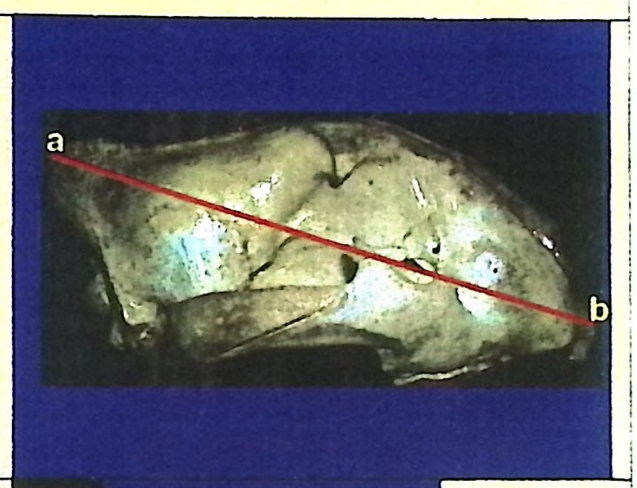
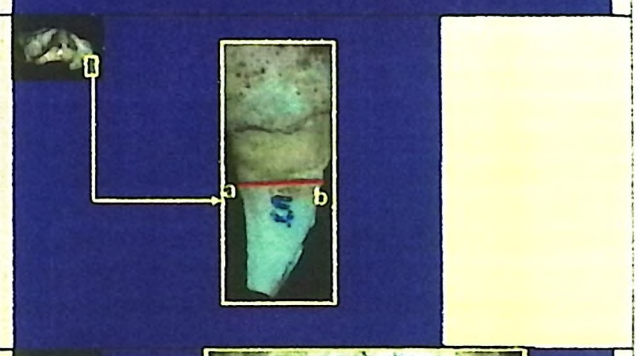
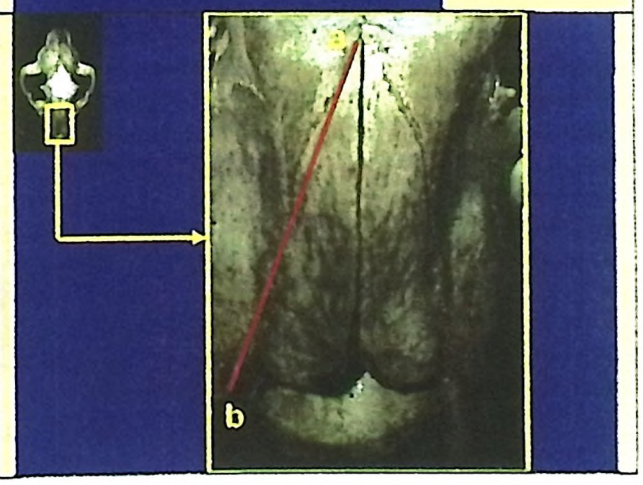


Figure 2.5. Cranial measurements for tiger and leopard skulls.

Table 2.9. Cranial measurements important for distinguishing tiger and leopard skulls.

1. Maximum zygomatic width	It measures the straight distance between the summits of the zygomatic arches. (a to b)	

2. Facial length	It measures from the point, which lies on the alveolar margin of the upper jaw in the mid-sagittal plane, projecting most anteriorly between the two central incisors. (a) to the point where the frontonasal suture meet the mid-sagittal plane. (b)	
3. Total skull length	It measures from the nuchal crest (a) to the anterior end of the premaxillary suture (b).	
4. C1 crown length	It measures transversely canine crown length (a to b).	
5. Nasal length (NL)	It measures overall length of the nasal bones (a to b).	

2.5.1.3. Statistical approach for distinguishing bones of tiger and leopard

Scapula measurements

Discriminant function analysis was performed using fourteen quantitative measurements of scapula of tiger (n=10) and leopard (n=11) (Table 2.3) through stepwise method in which each of the variables was entered in a row and assessed for its significance of classification function using F statistic (i.e., F to remove 3.14). Since several of the variables would show multi-collinearity, Wilk's lambda values were used to ascertain the degree of collinearity and remove those variables from further analyses. Fourth step variables were used in the analysis (Table 2.10).

A function could differentiate the tiger and leopard scapula bones absolutely. First function itself could explain 100% variability (Table 2.11). Four variables were used to derive a function (Table 2.12). The variables used in the function were as follows: height of scapula (HS), greatest dorsal length (LD), smallest length of the neck (SLC), and greatest length of scapula spine (GLSS).

Table 2.10. Variables used in the analysis.

Step	Variables	Tolerance	F to Remove	Wilks' Lambda
4	SLC	0.49	4.73	.15
	LD	0.18	24.50	.32
	HS	0.26	7.60	.17
	GLSS	0.64	5.21	.15

Table 2.11. Eigenvalues.

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	8.41	100.0	100.0	.95

a First 1 canonical discriminant functions were used in the analysis.

Table 2.12. Variables involved in the Function.

Variables	Function
	1
Height of scapula (HS)	1.30
Greatest dorsal length (LD)	-2.05
Smallest length of the neck (SLC)	.80
Greatest length of scapula spine (GLSS)	.73

The Discriminant function for scapula of tiger and leopard bones is given in Table 2.13. The coefficients of these four variables using Fisher's linear Discriminant functions were used to differentiate tiger and leopard bones. The classification formula is: $D_i = C_i + (V_1 \times U_1) + (V_2 \times U_2) + (V_3 \times U_3) + \ldots\ldots + (V_n \times U_n)$.

where D_i = Discriminate score, C_i =Constant, V_1 = Value of the 1^{st} variable measured of the i^{th} sample and U_1 = Fisher linear coefficient value of the i^{th} sample for the i^{th} species. The unknown sample was assigned to the species for which the discriminant score was the highest. Classification accuracy for these samples was 100%.

Table 2.13. Fisher's linear discriminant functions (Classification Function Coefficients).

Variables	Species	
	Tiger	Leopard
HS	1.17	.88
LD	-.66	-.46
SLC	1.92	1.23
GLSS	.54	.38
(Constant)	-188.80	-99.83

100% correctly classified

Pelvic measurements

Based on the twelve measurements of pelvic bones (Table 2.4) DFA was performed. First step variable was used in the analysis (Table 2.14). For this DFA eight samples of tiger and two samples of leopard pelvic were used.

Table 2.14. Variables used in the Analysis.

Step		Tolerance	F to Remove
1	GBTI	1.00	27.52

A function could differentiate the tiger and leopard pelvic bone absolutely. First function itself could explain 100% variability (Table 2.15). Only one variable was used to derive a function (Table2.16). The variable used in the function was greatest width across the tuber ischiae of pelvic (GBTI).

Table 2.15. Eigenvalues.

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	3.44	100.0	100.0	.88

Standardized Canonical Discriminant Function Coefficients

Table 2.16. Variable involved in the analysis.

Variable	Function
	1
GBTI	1.00

The discriminant function of pelvic bones of both species is given in Table 2.17. The coefficient of the variable using Fisher's linear Discriminant functions was used to differentiate tiger and leopard pelvic. The classification formula is: $D_i = C_i + (V_1 \times U_1)$

where D_i = Discriminate score, C_i =Constant, V_1 = Value of the 1^{st} variable measured of the i^{th} sample and U_1 = Fisher linear coefficient value of the i^{th} sample for the i^{th} species. The unknown sample was assigned to the species for which the discriminant score was the highest. Classification accuracy for these samples was 100%.

Table 2.17. Fisher's linear discriminant functions (Classification Function Coefficients).

Variable	SPECIES	
	Tiger	Leopard
GBTI	4.60	3.83
(Constant)	-301.46	-208.34

Differentiation of femur of tiger from leopard

Ten measurements were taken for femur bones of tiger and leopard (Table 2.6). Three dimensional plots indicate clear separation of femur of tiger from leopard using variables such as greatest length, inter-condylar distance and smallest width at diaphysis (Fig. 2.6).

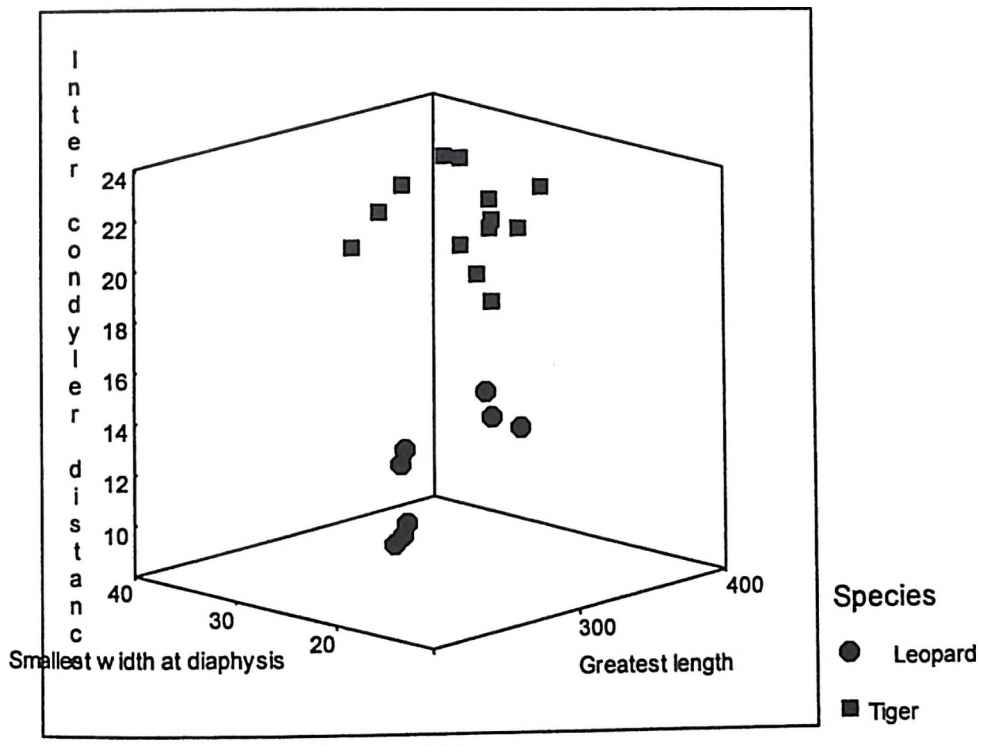


Figure 2. 6. Scatter plot showing differences in the measurements of tiger and leopard femur.

Differentiation of humerus of tiger and leopard

Table 2.18. Descriptive statistics of humerus of tiger.

Variables	N	Mean (mm)	Std. Error	Variance
GL	9	301.32	6.54	384.86
GL1	9	293.12	5.07	231.01
GLC	9	287.60	7.48	503.63
WP	9	73.39	1.46	19.18
DP	9	53.34	6.62	394.92
SD	9	28.79	1.35	16.29
WD	9	75.83	1.25	14.01
WT	9	52.40	1.24	13.86
DD	9	25.63	.54	2.65
DFP	9	182.64	4.40	173.97

Table 2.19. Descriptive statistics of humerus of leopard.

Variables	N	Mean(mm)	Std. Error	Variance
GL	5	220.74	5.16	132.96
GL1	5	217.37	5.49	150.57
GLC	5	215.30	7.06	248.94
WP	5	43.48	1.83	16.76
DP	5	39.44	7.15	255.27
SD	5	18.79	1.09	5.91
WD	5	50.17	2.03	20.56
WT	5	34.83	.98	4.77
DD	5	19.60	.68	2.30
DFP	5	124.46	1.69	14.26

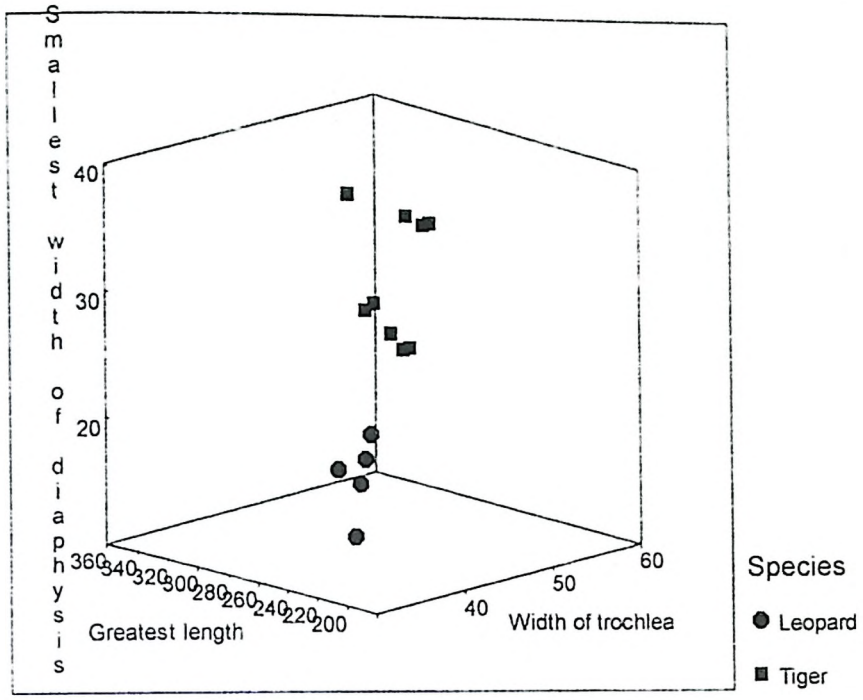
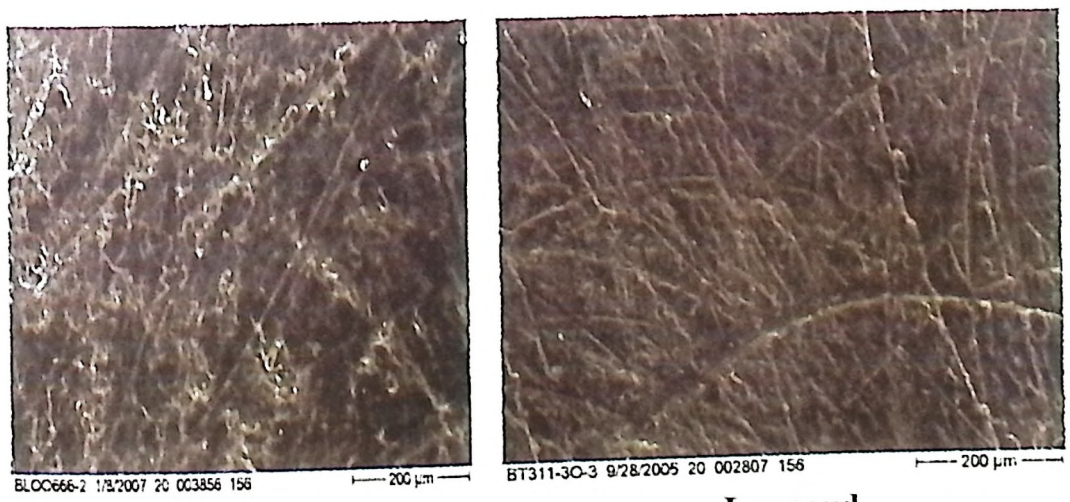


Figure 2.7. Scatter plot indicates differences between humerus of tiger and leopard.

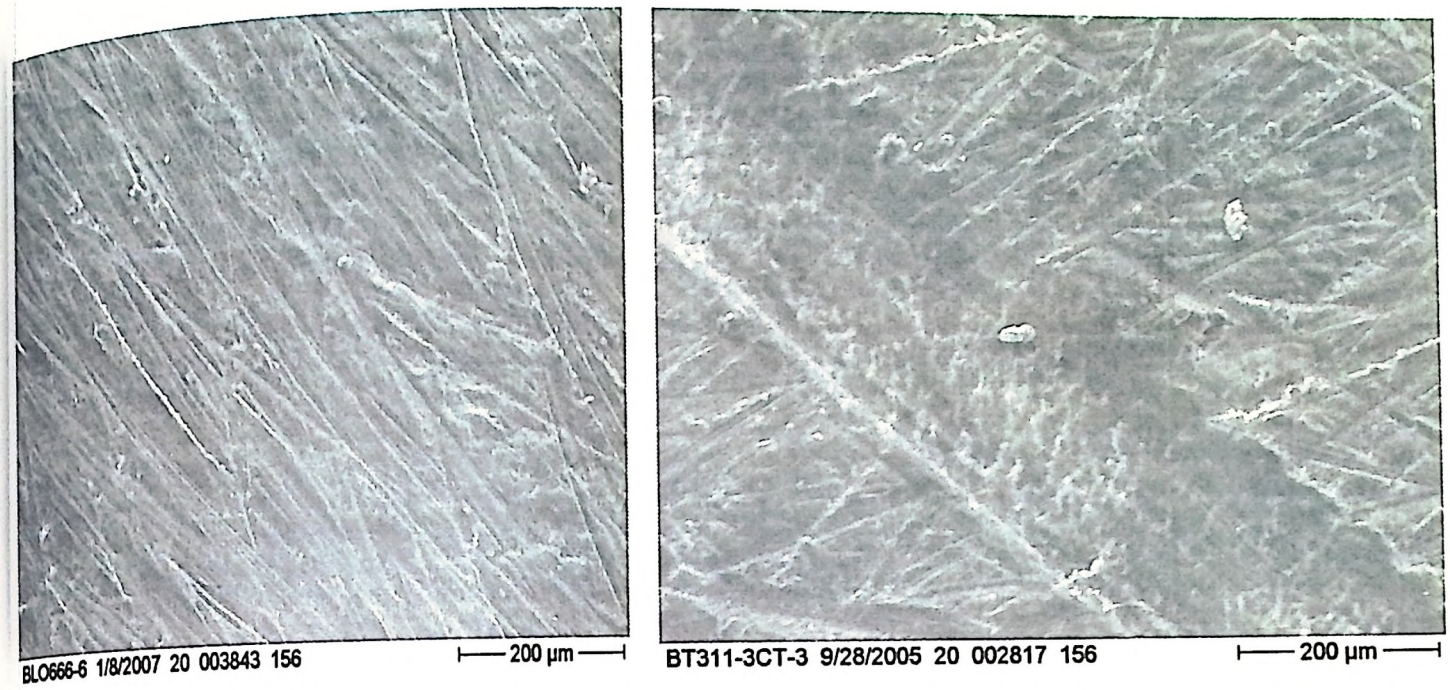
The descriptive statistic in Table 2.18 and 2.19 gives the differences in various measurements of humerus of tiger and leopard. Scatter plots using greatest length, width of trochlea and smallest width of diaphysis could differentiate between tiger and leopard humerus bone (Fig. 2.7).

2.5.2. Scanning electron microscopic characteristics



Tiger Leopard

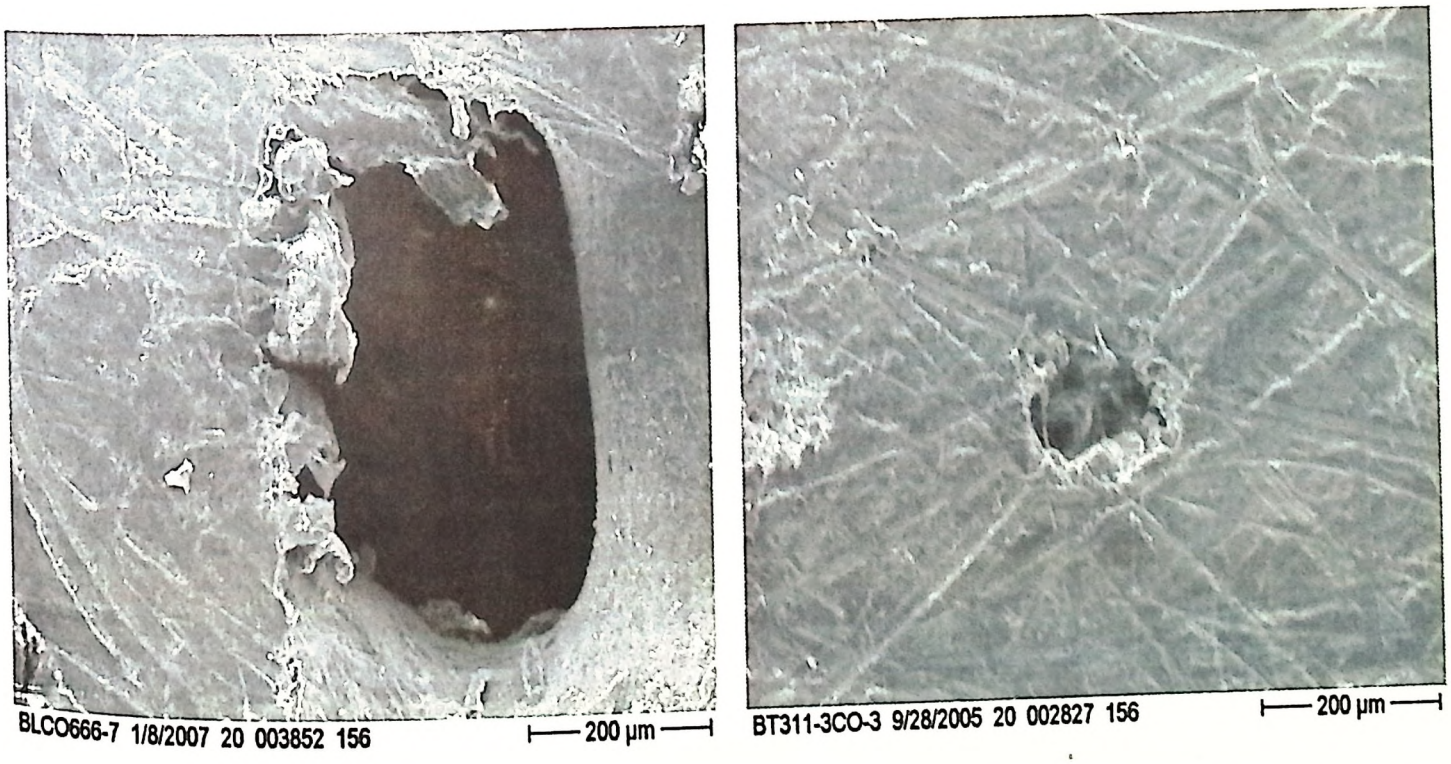
Figure 2.8. Scanning micrographs to distinguish outer portion of tiger and leopard femur.



Tiger **Leopard**

Figure 2.9. Scanning micrographs of cortex portion of tiger and leopard femur.

Observations at the outer portion of tiger and leopard bones show that leopard bone had much smoother surface as compared to the tiger bone. There were presence of fine lineation in the micrograph of leopard outer portion where as tiger had thick lineation (Fig. 2.8). Observations at cortex portion of bones indicate oblique lineation in tiger (Fig. 2.9).

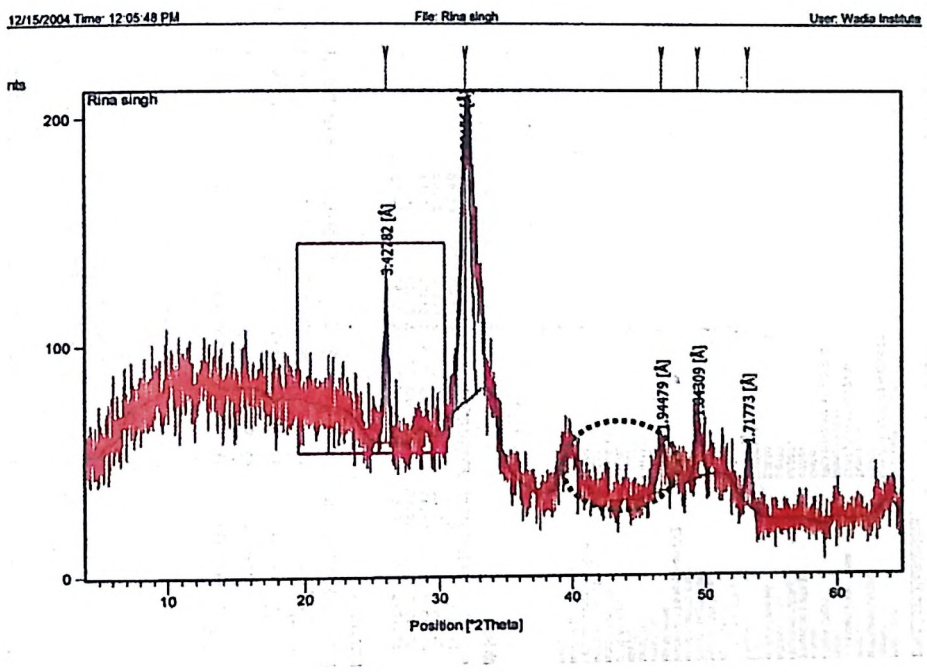


Tiger **Leopard**

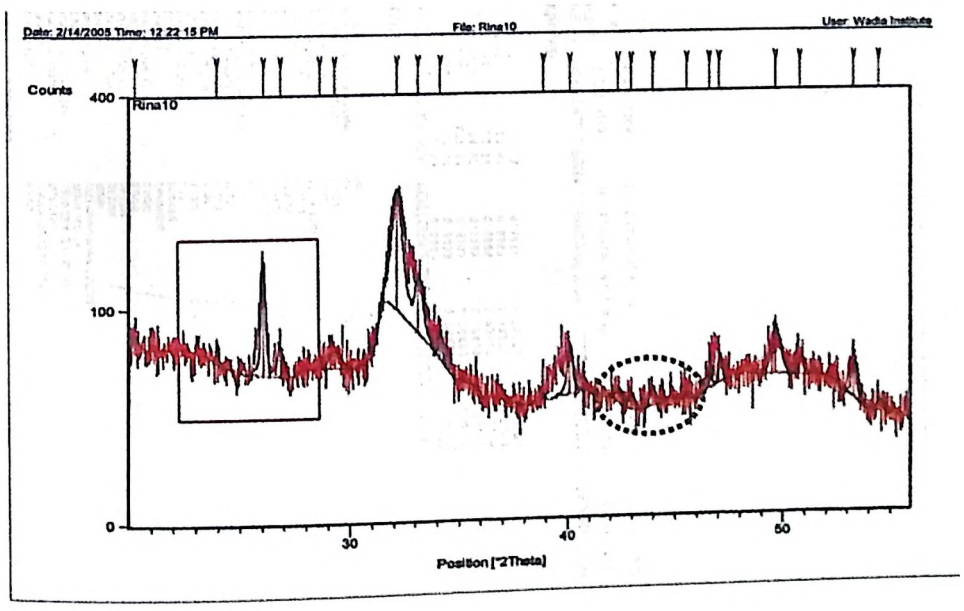
Figure 2.10. Scanning micrographs of core portion of tiger and leopard femur.

Scan micrograph of tiger and leopard at core region illustrates that distinct differences are present as in tiger core area there were numerous oblong holes (length 5000 μm and width 3000 μm) only one hole was visible in the micrograph (Fig. 2.10). In leopard the holes were smaller and not oblong (length 1000 μm and width 1500 μm).

2.5.3. X-ray diffraction characteristics



Tiger



Leopard

Figure 2.11. X-ray diffractogram to distinguish bone of tiger and leopard.

Bone samples of tiger (n=5) and leopard (n=5) were analyzed using X-ray diffraction technique. Two shoulder peaks were noticed in some leopard diffractogram at 25 and 26 ^{0} but, it was not consistent in all the diffractogram. Few peaks were noticed between 42-44^0 angles in leopard bones diffractogram, these were absent in tiger diffractogram. However no clear demarcation was found in the diffractograms of tiger and leopard (Fig. 2.11).

2.5.4. Thermo gravimetric characteristics

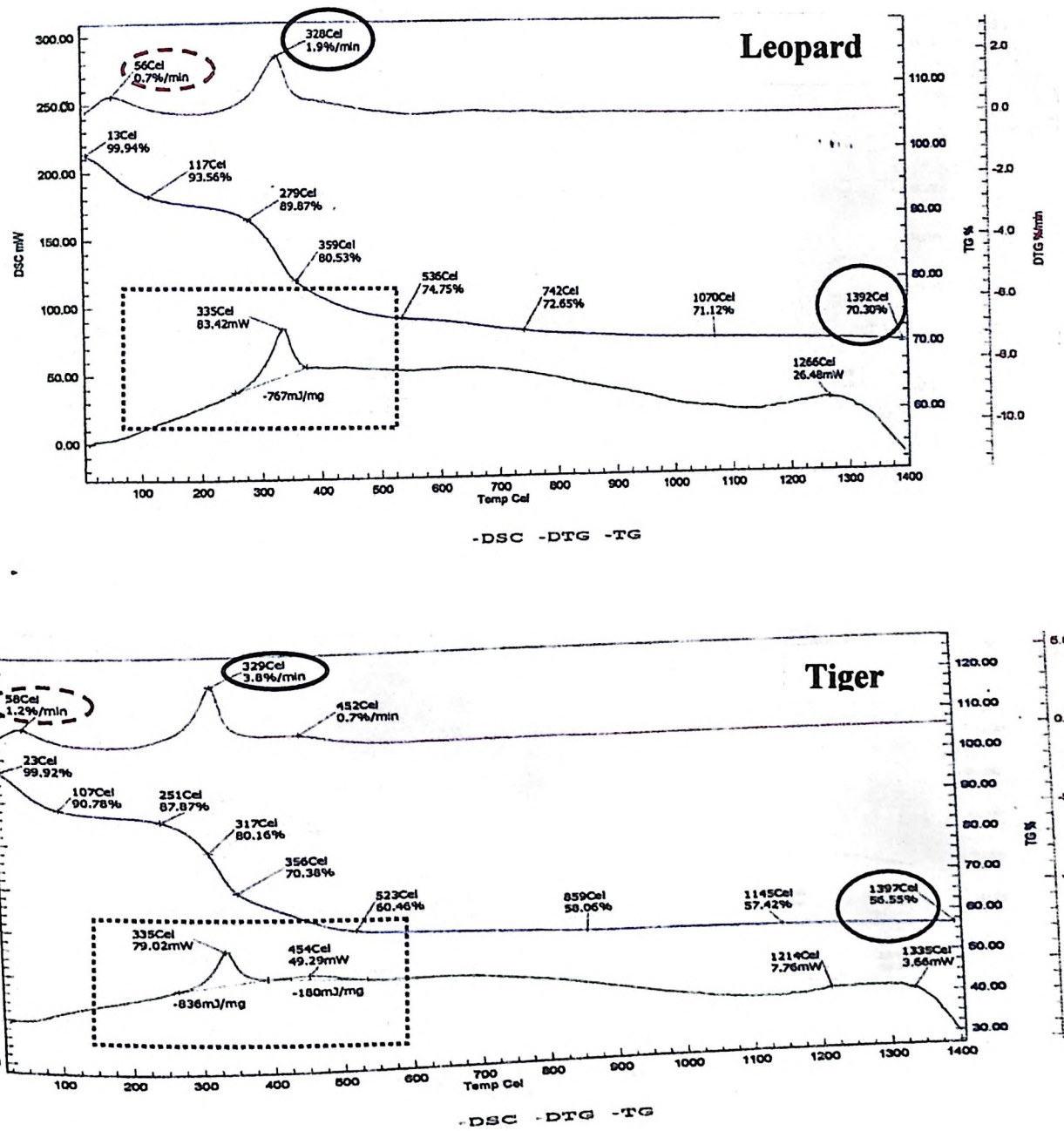


Figure 2.12. Thermographs of tiger and leopard bones.

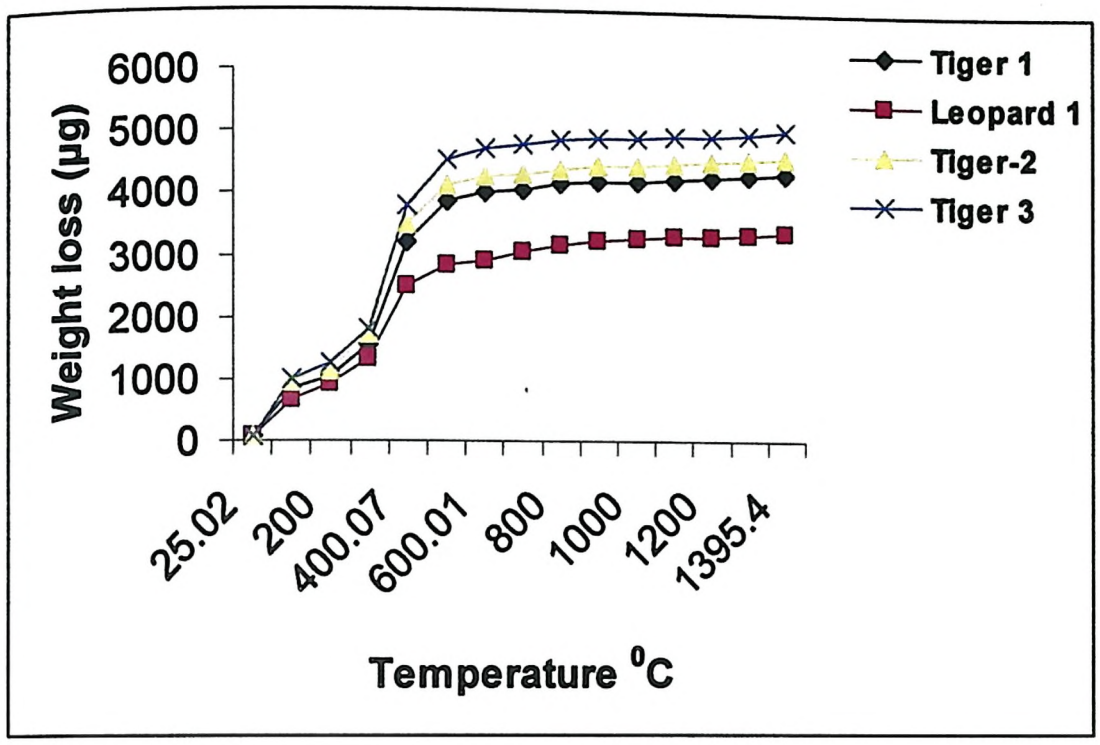


Figure 2.13. Weight loss in relation to temperature in tiger and leopard bones.

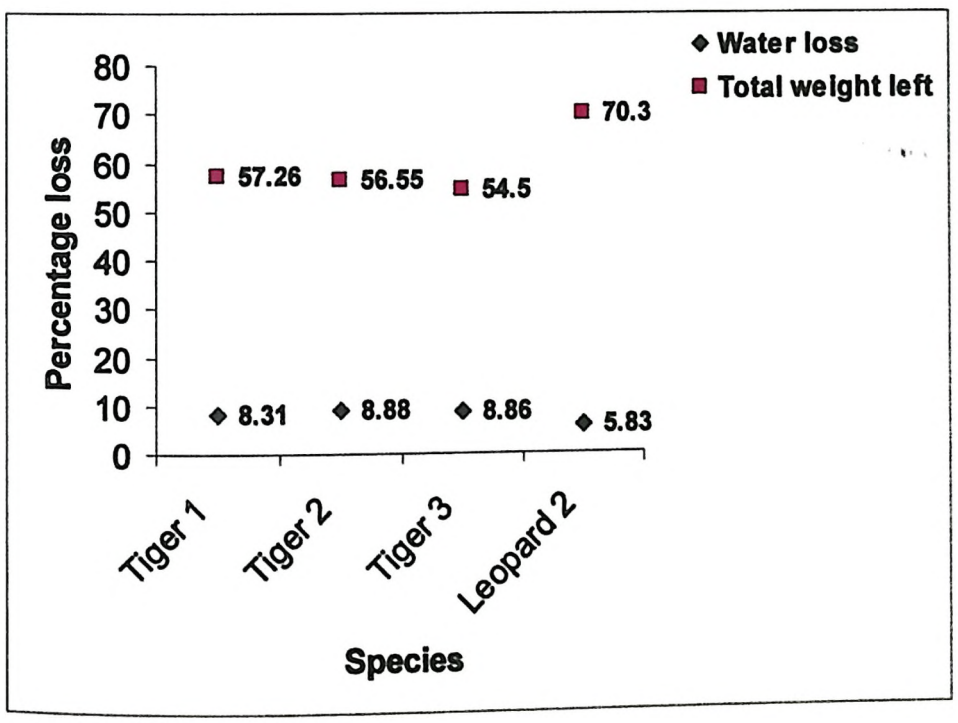


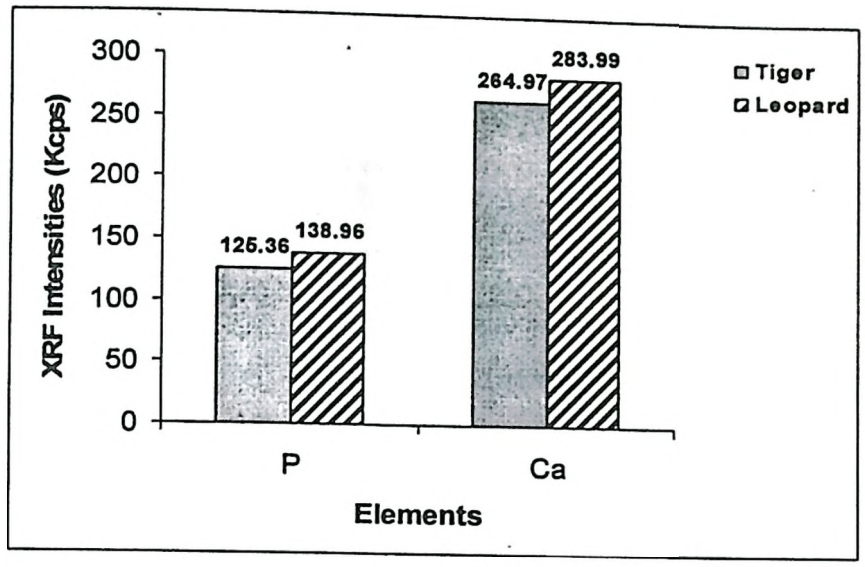
Figure 2.14. Percent water loss at 100 ^{0}C and total weight left at 1400 ^{0}C.

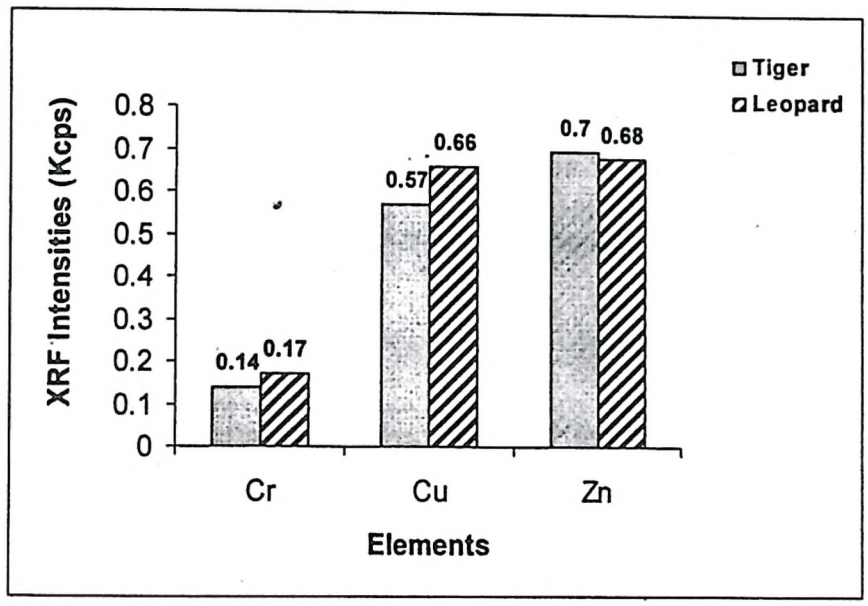
Thermograph of leopard (n=1) and tiger (n=3) were distinctly different although both had two major peaks, but the rate of loss of weight was different at the peaks. In leopard, first peak was at 56 ^{0} C, and rate of loss was 0.7%/min where as rate of loss at 58 ^{0} C was 1.2%/min in tiger. At the second peak, rate of loss in leopard was 1.9%/min at 328 ^{0} C, where as this was 3.8%/min at 329 ^{0} C in tiger. There was only one peak of energy loss of 767 mj/mg at 335 ^{0} C in leopard but, in tiger there were two peaks of energy loss of 836 mj/mg at 335 ^{0} C and 180 mj/mg at 454 ^{0}C. Total weight left at 1392 ^{0} C was 70.30% in leopard but it was 56.55% in tiger at 1397 ^{0} C (Fig. 2.12). The nature of weight loss pattern indicates that maximum weight loss in both the bones were noticed in between 300 to 500 ^{0} C. The weight loss was always lower in leopard as compared to tiger. At 500^0 C, the weight loss in leopard was 2829.16 µg, where as in tiger it was 4119.53 µg (Fig. 2.13). The water loss percentage was approximately 8% in tiger where as in leopard it was 5%. Differences in weight left at 1400 ^{0} C, was also noted in leopard (70.30%) in leopard and in tiger (56.55%).

2.5.5. Elemental analysis

2.5.5.1 X-ray intensities of various elements based on XRF

Slight differences were noted in XRF intensities of calcium and phosphorous. Major differences were there in intensities of sodium, aluminum, silicon, chlorine and iron and these were higher in tiger except for iron (Fig. 2.15).





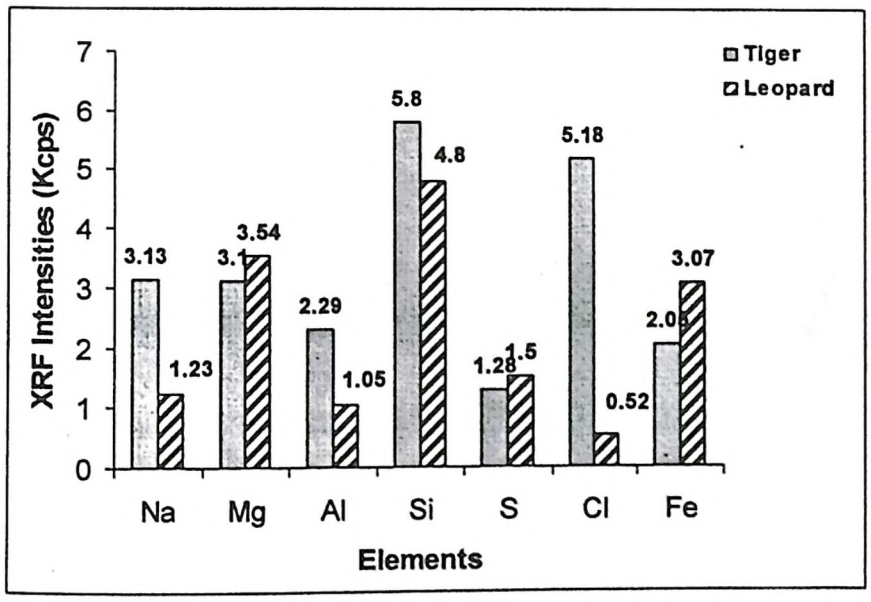
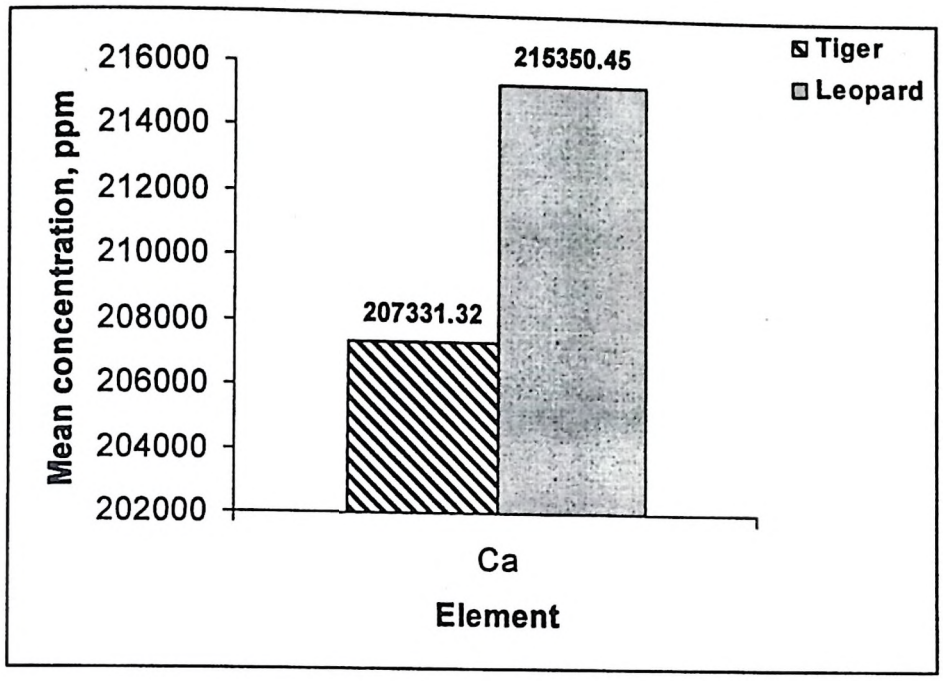
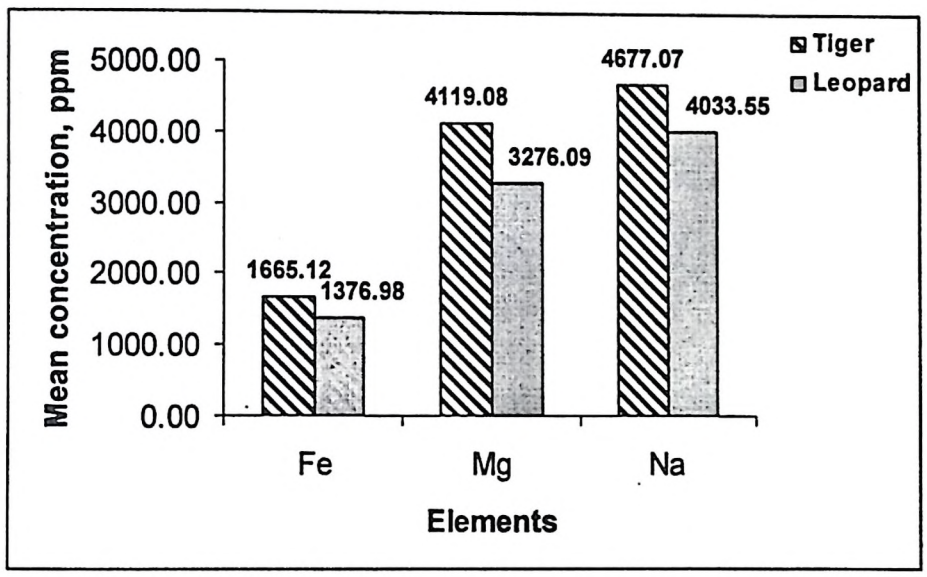


Figure 2.15. X-ray intensities (KCps) of various elements in tiger and leopard bones.

2.5.5.2 Inductively coupled plasma – mass spectrometry (ICP-MS)





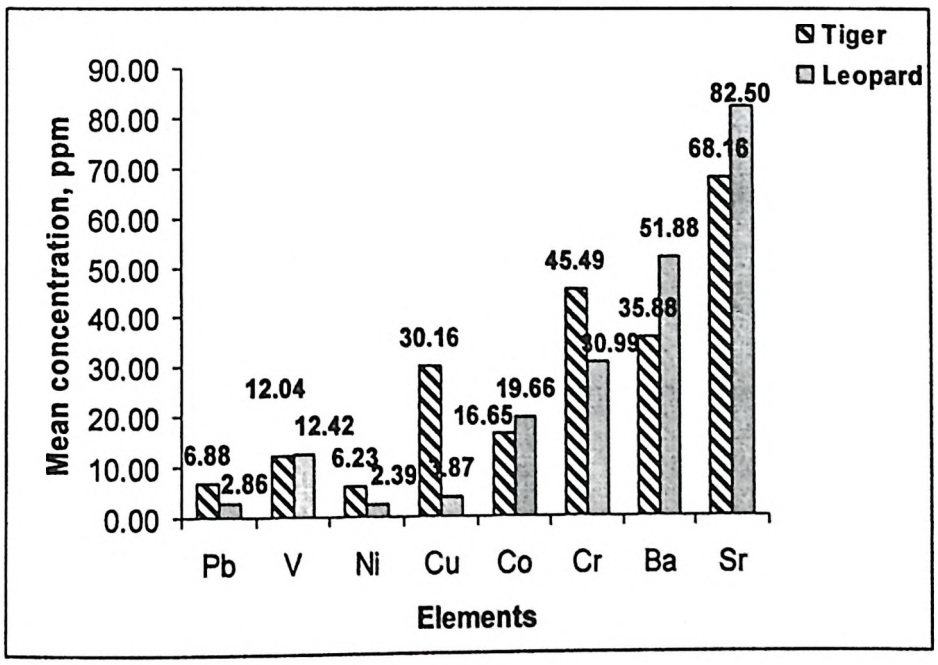


Figure 2.16. Mean Concentration of Ca, Fe, Mg, Na, Pb, V, Ni, Cr, Co, Cu, Ba and Sr in bones of tiger and leopard.

Analysis indicate that the mean concentration of calcium (Ca), barium (Ba), cobalt (Co) and strontium (Sr) were more in leopard than tiger bones. Tiger has higher concentrations of iron (Fe), magnesium (Mg), sodium (Na), lead (Pb), nickel (Ni), copper (Cu), and chromium (Cr) than leopard (Fig. 2.16). Of the eighteen elements examined, major differences detected was in Ca, Cu, Cr, Ba, Sr, Fe, Mg and Na.

Discriminant Function Analysis

Third step variables were used in the analysis (Table 2.20).

Table 2.20. Variables used in the analysis.

Step	Variables	Tolerance	F to Remove	Wilks' Lambda
3	Ca	.003	3347.21	.38
	Cu	.002	552.22	.06
	Na	.006	160.87	.02

A function could differentiate the tiger and leopard bones absolutely. First function itself could explain 100% variability (Table 2.21). Three variables were used to derive a function (Table2.22). The variables used in the function were calcium (Ca), copper (Cu) and sodium (Na) concentrations.

Table 2.21. Eigenvalues.

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	8735.42	100.0	100.0	1.00

Standardized Canonical Discriminant Function Coefficients

Table 2.22. Variables involve in function.

Variables	Function
	1
Ca	-19.57
Na	12.66
Cu	22.20

The discriminant function of both the bones is given in Table 2.23. The coefficients of these three variables using Fisher's linear Discriminant functions were

used to differentiate tiger and leopard bones. The classification formula is: $D_i = C_i + (V_1 \times U_1) + (V_2 \times U_2) + (V_3 \times U_3)$.

where D_i = Discriminate score, C_i =Constant, V_1 = Value of the 1st variable measured of the i^{th} sample and U_1 = Fisher linear coefficient value of the i^{th} sample for the i^{th} species. The unknown sample was assigned to the species for which the discriminant score was the highest. Classification accuracy for these samples was found to be 100%.

Table 2.23. Fisher's linear discriminant functions (Classification Function Coefficients).

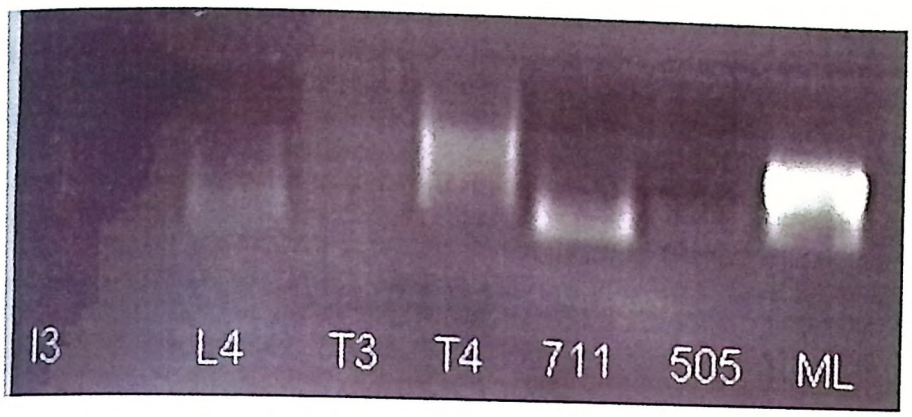
Variables	Bones	
	Tiger	Leopard
Ca	39.97	42.07
Na	-65.06	-68.48
Cu	-2034.03	-2140.89
(Constant)	-3960357.60	-4387121.12

Classification accuracy 100%

2.5.6. DNA techniques

Extraction and PCR amplification

Although, it is difficult to extract DNA from hard tissues but, fairly goods amount of DNA (300-400bp) were extracted from bones of tiger and leopard using Gene clean and Bio Robotic methods as per their protocols provided by manufacturer (Fig.2.17). These two methods can be used for extraction successfully.



Gene clean method

Bio Robotic method

Figure 2.17. DNA extraction from bones of tiger and leopard.

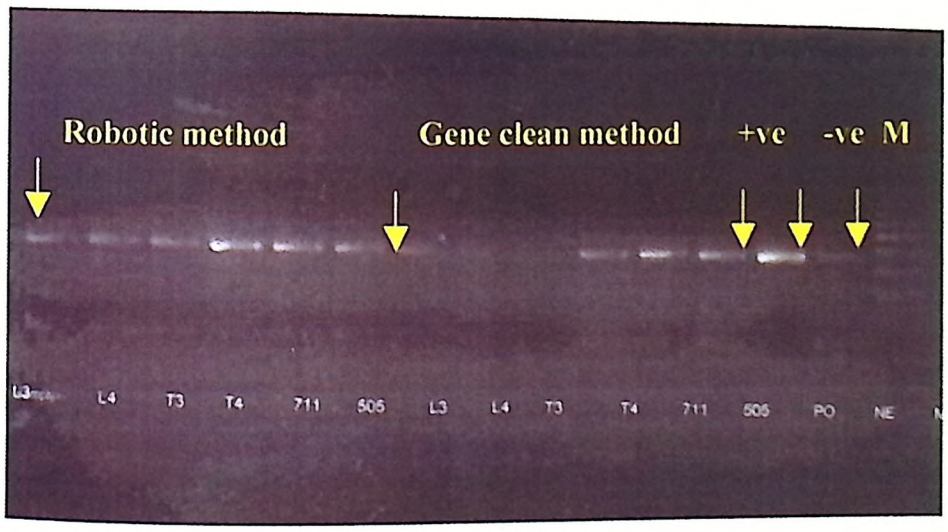


Figure 2.18. PCR of DNA extracted from both the above methods.

PCR of DNA samples extracted from bones of tiger and leopard were amplified successfully (Fig. 2.18) Gel purification of PCR products was needed for further sequencing and was performed.

Sequencing

Sequencing of bone sample using Cytb 381 primer yielded 328bp. Though, the quality of sequence was not goods. PCR was also done with 16S rRNA primer yielded 550 bp products (Fig. 2.19). Sequence was improved by cleaning the PCR product. Further these sequences were matched with NCBI data and checked for the species. Alignment of tiger and leopard sequences were done to find out the polymorphism.

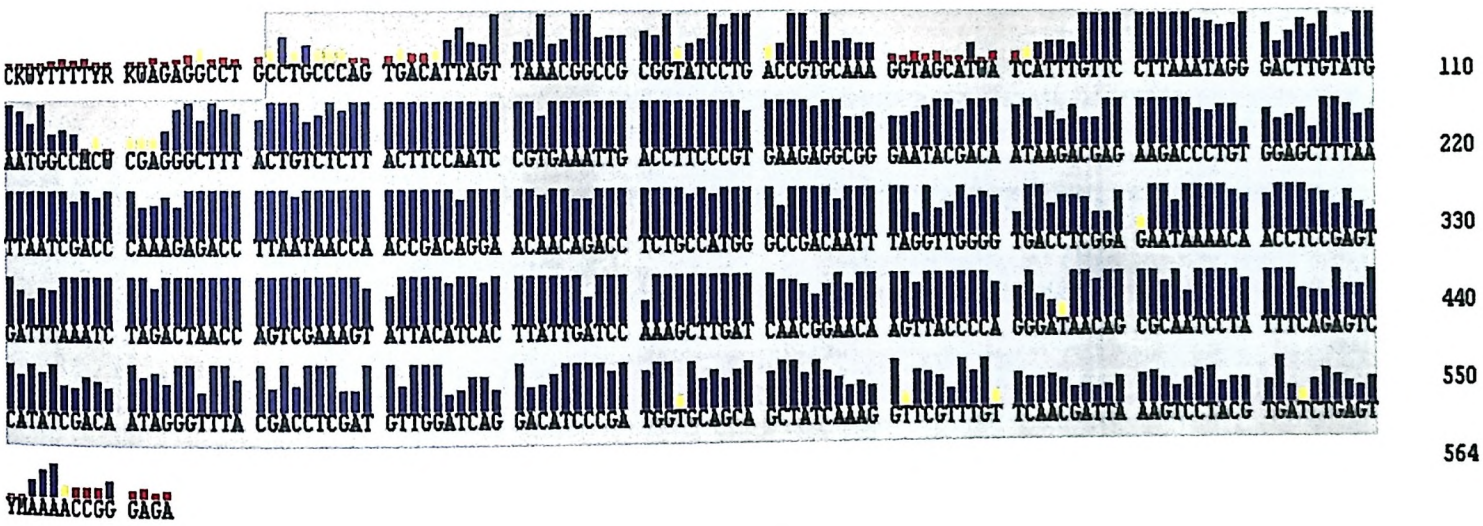


Figure 2.19. Sequence of tiger using 16S rRNA primer.

```
                                    10        20        30        40        50
                                    ....|....|....|....|....|....|....|....|....|....|
AY297928 Panthera pardus      1     AGGAGCTACTGTCATCACCAACCTCCTATCAGAAATCCCCTGCATCGGAA
AY297927 Panthera pardus      1     ..................................................
AY297926 Panthera pardus      1     ..................................................
AY297925 Panthera pardus      1     ..................................................
AY736656 Panthera tigris      1     ...G..A..C..A.............G....C......A.AT..T..G.
AY736655 Panthera tigris      1     ...G..A..C..A.............G....C......A.AT..T..G.
AY736654 Panthera tigris      1     ...G..A..C..A.............G....C......A.AT..T..G.
AY736653 Panthera tigris      1     ...G..A..C..A.............G....C......A.AT..T..G.

                                    60        70        80        90        100
                                    ....|....|....|....|....|....|....|....|....|....|
AY297928 Panthera pardus      51    CTAATTTAGTAGAATACATCTGAGGAGGATTCTCAGTAGACAAAGCCACC
AY297927 Panthera pardus      51    ..................................................
AY297926 Panthera pardus      51    ..................................................
AY297925 Panthera pardus      51    ..................................................
AY736656 Panthera tigris      51    .CG.CC.......G.GA........G..T................T...
AY736655 Panthera tigris      51    .CG.CC.......G.GA........G..T................T...
AY736654 Panthera tigris      51    .CG.CC.......G.GA........G..T................T...
AY736653 Panthera tigris      51    .CG.CC.......G.GA........G..T................T...

                                    110       120       130       140       150
                                    ....|....|....|....|....|....|....|....|....|....|
AY297928 Panthera pardus      101   CTGACACGATTCTTTGCCTTTCGCTTTATTCTCCCATTTATTATTTCAGC
AY297927 Panthera pardus      101   ..................................................
AY297926 Panthera pardus      101   ..................................................
AY297925 Panthera pardus      101   ..................................................
AY736656 Panthera tigris      101   ..................C.A...C..C..T........CG.C.....
AY736655 Panthera tigris      101   ..................C.A...C..C..T........CG.C.....
AY736654 Panthera tigris      101   ..................C.A...C..C..T..G.....CG.C.....
AY736653 Panthera tigris      101   ..................C.A...C..C..T..G.....CG.C.....

                                    160       170       180       190       200
                                    ....|....|....|....|....|....|....|....|....|....|
AY297928 Panthera pardus      151   CCTAGCAGCAGTCCACCTTTCATTCCTACACCAAACAGGACCTAACAACC
AY297927 Panthera pardus      151   ..................................................
AY297926 Panthera pardus      151   ..................................................
AY297925 Panthera pardus      151   ..................................................
AY736656 Panthera tigris      151   ................CCT......T...G........T.C..T....
AY736655 Panthera tigris      151   ................CCT......T...G........T.C..T....
AY736654 Panthera tigris      151   ................CCT......T...G........T.C..T....
AY736653 Panthera tigris      151   ................CCT......T...G........T.C..T....

                                    210       220       230       240       250
                                    ....|....|....|....|....|....|....|....|....|....|
AY297928 Panthera pardus      201   CCTCAGGAATAGTATCTGACTTGGATAAAATCCCATTCCACTCATACTAC
AY297927 Panthera pardus      201   ..................................................
AY297926 Panthera pardus      201   ..................................................
AY297925 Panthera pardus      201   ..................................................
AY736656 Panthera tigris      201   ..........G..G..C....CA..C..............C........
AY736655 Panthera tigris      201   ..........G..G..C....CA..C..............C........
AY736654 Panthera tigris      201   ..........G..G..C....CA..C..............C........
AY736653 Panthera tigris      201   ..........G..G..C....CA..C..............C........

                                    260       270       280       290       300
                                    ....|....|....|....|....|....|....|....|....|....|
AY297928 Panthera pardus      251   ACAATCAAAGACATCCTGGGTCTTTTATTCCTAATCCGAATCCTACTTGT
AY297927 Panthera pardus      251   ..................................................
AY297926 Panthera pardus      251   ..................................................
AY297925 Panthera pardus      251   ..................................................
AY736656 Panthera tigris      251   .....T.....T...T.A..C..C...G.A.......T..C...CACAC.
AY736655 Panthera tigris      251   .....T.....T...T.A..C..C...G.A.......T..C...CACAC.
AY736654 Panthera tigris      251   .....T.....T...T.A..C..C...G.A.......T..C...CA.AC.
AY736653 Panthera tigris      251   .....T.....T...T.A..C..C...G.A.......T..C...CA.AC.
```

```
                                                  310
                                          ....|....|....|....
AY297928 Panthera pardus    301 CCTATTTTTCACCTCACCT
AY297927 Panthera pardus    301 ...................
AY297926 Panthera pardus    301 ...................
AY297925 Panthera pardus    301 ...................
AY736656 Panthera tigris    301 A..CG.CC.ATT......A
AY736655 Panthera tigris    301 A..CG.CC.ATT......A
AY736654 Panthera tigris    301 A..CG.CC.ATT......A
AY736653 Panthera tigris    301 A..CG.CC.ATT......A
```

Figure 2.20. Alignment of DNA sequences of cytochrome b of mitochondrial gene *Panthera tigris* and *Panthera pardus*.

```
                                        10        20        30        40        50
                                ....|....|....|....|....|....|....|....|....|....|
AF006459 Panthera tigris    1   AAATAGGGACTTGTATGAATGGCCACAAGAGGGCTTTACTGTCTCTTACT
AY452106 Panthera tigris    1   ............................C.....................
AY452110 Panthera tigris    1   ............................C.....................
AY452109 Panthera tigris    1   ............................C.....................
AF006443 Panthera pardus    1   ............................C.....................
AY452108 Panthera pardus    1   ............................C.....................
EF202838 Panthera pardus    1   ............................C.....................

                                        60        70        80        90       100
                                ....|....|....|....|....|....|....|....|....|....|
AF006459 Panthera tigris    51  TCCAATCCGTGAAATTGACCTTCCCGTGAAGAGGCGGGAATATGACAATA
AY452106 Panthera tigris    51  ..................................................
AY452110 Panthera tigris    51  ..........................................C.......
AY452109 Panthera tigris    51  ..................................................
AF006443 Panthera pardus    51  ...G..............................................
AY452108 Panthera pardus    51  ...G..............................................
EF202838 Panthera pardus    51  ...G..............................................

                                       110       120       130       140       150
                                ....|....|....|....|....|....|....|....|....|....|
AF006459 Panthera tigris    101 AGACGAGAAGACCCTATGGAGCTTTAATTAACCGACCCAAAGAGACCTTA
AY452106 Panthera tigris    101 ..................................................G
AY452110 Panthera tigris    101 ..............G.................T..................
AY452109 Panthera tigris    101 ..................................................G
AF006443 Panthera pardus    101 ..............................................T....
AY452108 Panthera pardus    101 ..............................................T....
EF202838 Panthera pardus    101 ..............................................T....
```

```
                                    160       170       180       190       200
                                    ....|....|....|....|....|....|....|....|....|....|
AF006459 Panthera tigris    151 ATAATCAACCGACAGGGATAACAAACCTCTACCATGGGTCGACAATTTAG
AY452106 Panthera tigris    151 ....................................................
AY452110 Panthera tigris    151 ....C.....A.C....G......G.......C.....
AY452109 Panthera tigris    151 ....................................................
AF006443 Panthera pardus    151 .C..CT......T.....C.............G.....
AY452108 Panthera pardus    151 .C..CT......T.....C.....
EF202838 Panthera pardus    151 .C..CT......T.....C.....

                                    210       220       230       240       250
                                    ....|....|....|....|....|....|....|....|....|....|
AF006459 Panthera tigris    201 GTTGGGGTGACCTCGGAGAATAAAACAACCTCCGAGTGATTTAAATCTAG
AY452106 Panthera tigris    201 ....................................................
AY452110 Panthera tigris    201 ....................................................
AY452109 Panthera tigris    201 ....................................................
AF006443 Panthera pardus    201 ....................................................
AY452108 Panthera pardus    201 ....................................................
EF202838 Panthera pardus    201 ....................................................

                                    310       320       330       340       350
                                    ....|....|....|....|....|....|....|....|....|....|
AF006459 Panthera tigris    301 ACGGAACAAGTTACCCTAGGGATAACAGCGCAATCCTATTTCAGAGTCCA
AY452106 Panthera tigris    301 ....................................T.....
AY452110 Panthera tigris    300 ..............C.....................C.....
AY452109 Panthera tigris    301 ....................................T.....
AF006443 Panthera pardus    301 ....................................T.....
AY452108 Panthera pardus    301 ....................................T.....
EF202838 Panthera pardus    301 ....................................T.....

                                    360
                                    ....|....|..
AF006459 Panthera tigris    351 TATCGACAATAG
AY452106 Panthera tigris    351 ............
AY452110 Panthera tigris    350 ............
AY452109 Panthera tigris    351 ............
AF006443 Panthera pardus    351 ............
AY452108 Panthera pardus    351 ............
EF202838 Panthera pardus    351 ............
```

Figure 2.21. Alignment of DNA sequences of 16S rRNA of mitochondrial gene of *Panthera tigris* and *Panthera pardus*.

Analysis of sequences revealed that more then 20% sites were polymorphic in cytochrome b gene and 5.52% sites were polymorphic in 16S rRNA region. Sixty-nine different sites were observed in 319bp of cytochrome b alignment region (Fig. 2.20) where as 20 different sites were observed in 362 bp of 16S rRNA region (Fig. 2.21).

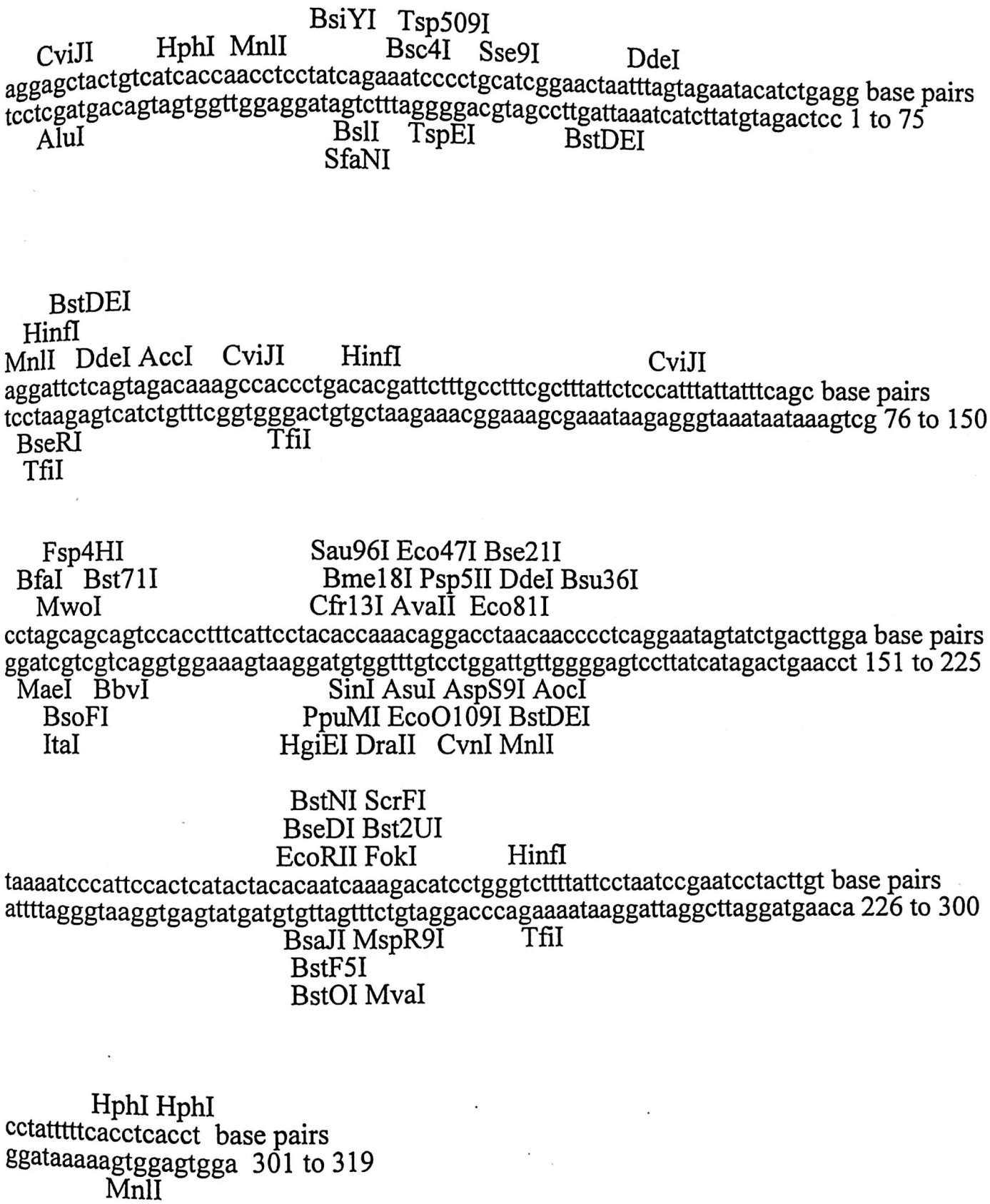


Figure 2.22. Restriction enzyme map of leopard cytochrome b gene generated using Webcutter 2.0.

BsiYI Bme18I AspS9I
BslI Cfr13I Eco47I BfaI BstDEI
HphI MnlI Bsc4I Van91I AvaII MaeI DdeI
aggggcaaccgtaatcaccaacctcctgtcagcaatcccatatattgggaccgacctagtagagtgaatctgagg base pairs
tccccgttggcattagtggttggaggacagtcgttagggtatataaccctggctggatcatctcacttagactcc 1 to 75
AccB7I Sau96I NlaIV HinfI
PflMI SinI AsuI BsmFI TfiI
Esp1396I HgiEI PspN4I

BsmBI
Alw26I
MnlI DdeI AccI CviJI HinfI BstF5I DdeI
gggtttctcagtagacaaagctaccctgacacgattctttgccttccacttcatccttccgtttatcgtctcagc base pairs
cccaaagagtcatctgtttcgatgggactgtgctaagaaacggaaggtgaagtaggaaggcaaatagcagagtcg 76 to 150
BstDEI AluI TfiI FokI BstDEI
BsmAI
Esp3I

Fsp4HI MboI Bsp143I AlwI CvnI
MwoI BstX2I XhoII AclWI AocI BstDEI
CviJI BsoFI MnlI BstYI BamHI PspN4I BstDEI HinfI
cctagcagcagtccacctcctattccttcacgaaacaggatccaataacccctcaggaatggtgtccgactcaga base pairs
ggatcgtcgtcaggtggaggataaggaagtgctttgtcctaggttattggggagtccttaccacaggctgagtct 151 to 225
MaeI BbvI DpnII MflI NlaIV Bse21I DdeI
BfaI Bst71I NdeII BstI DpnI DdeI Bsu36I PleI
ItaI Sau3AI Kzo9I Eco81I MnlI

MseI HaeIII BstDEI Eco255I
Tsp509I DdeI AatI BsuRI Csp6I
Sse9I Eco32I Pme55I DdeI Acc113I MnlI
caaaatcccattccacccatactacacaattaaagatatcttaggcctcttagtactaatcctaaccctcatact base pairs
gttttagggtaaggtgggtatgatgtgttaatttctatagaatccggagaatcatgattaggattgggagtatga 226 to 300
TspEI EcoRV StuI Eco147I ScaI
Tru9I BstDEI PalI MnlI RsaI
Tru1I CviJI SseBI AfaI

HphI
actcgtcctattctcacca base pairs
tgagcaggataagagtggt 301 to 319

Figure 2.23. Restriction enzyme map of tiger cytochrome b gene generated using Webcutter 2.0.

Table 2.24. Restriction enzymes which are cutting partial fragment of cyochrome b gene of tiger and leopard at different sites.

Enzyme name	Recognition sequence	*Panthera tigris*		*Panthera pardus*	
		No. of cuts	Positions of sites	No. of cuts	Positions of sites
AatI	**agg/cct**	**1**	**270**	**-**	**-**
Acc113I	**agt/act**	**1**	**279**	**-**	**-**
AccB7I	**ccannnn/ntgg**	**1**	**44**	**-**	**-**
AclWI	**ggatc**	**1**	**192**	**-**	**-**
AfaI	**gt/ac**	**1**	**279**	**-**	**-**
AluI	ag/ct	1	95	1	5
Alw26I	**gtctc**	**1**	**147**	**-**	**-**
AlwI	**ggatc**	**1**	**192**	**-**	**-**
AspS9I	g/gncc	1	48	1	188
AsuI	g/gncc	1	48	1	188
AvaII	g/gwcc	1	48	1	188
BamHI	**g/gatcc**	**1**	**188**	**-**	**-**
BfaI	c/tag	2	56 152	1	152
Bme18I	g/gwcc	1	48	1	188
BsaJI	*c/cnngg*	-	-	*1*	*265*
BseDI	*c/cnngg*	-	-	*1*	*265*
BseRI	*gaggag*	-	-	*1*	*77*
Bsp143I	**/gatc**	**1**	**188**	**-**	**-**
Bst2UI	*cc/wgg*	-	-	*1*	*266*
BstDEI	c/tnag	7	70 82 145 202 220 265 274	3	70 82 202
BstF5I	ggatg	1	131	1	266
BstI	**g/gatcc**	**1**	**188**	**-**	**-**
BstNI	*cc/wgg*	-	-	*1*	*266*
BstOI	*cc/wgg*	-	-	*1*	*266*
BstX2I	**r/gatcy**	**1**	**188**	**-**	**-**
BstYI	**r/gatcy**	**1**	**188**	**-**	**-**
BsuRI	**gg/cc**	**1**	**270**	**-**	**-**
Cfr13I	g/gncc	1	48	1	188
Csp6I	**g/tac**	**1**	**278**	**-**	**-**
CviJI	rg/cy	3	95 149 270	3	5 95 149
DdeI	c/tnag	7	70 82 145 202 220 265 274	3	70 82 202
DpnI	**ga/tc**	**1**	**190**	**-**	**-**
DpnII	**/gatc**	**1**	**188**	**-**	**-**
DraII	rg/gnccy	1	270	1	188
Eco255I	**agt/act**	**1**	**279**	**-**	**-**
Eco32I	**gat/atc**	**1**	**262**	**-**	**-**
Eco47I	g/gwcc	1	48	1	188

EcoRV	**gat/atc**	**1**	**262**	-	-
Esp1396I	**ccannnn/ntgg**	**1**	**44**	-	-
Esp3I	**cgtctc**	**1**	**147**	-	-
EcoO109I	*rg/gnccy*	-	-	*1*	*188*
EcoRII	*/ccwgg*	-	-	*1*	*264*
FokI	ggatg	1	131	1	266
HaeIII	**gg/cc**	**1**	**270**	-	-
HgiEI	g/gwcc	1	48	1	188
HinfI	g/antc	3	66 108 218	3	78 108 288
HphI	ggtga	2	19 318	3	19 313 318
Kzo9I	**/gatc**	**1**	**188**	-	-
MaeI	c/tag	2	56 152	1	152
MboI	**/gatc**	**1**	**188**	-	-
MflI	**r/gatcy**	**1**	**188**	-	-
MnlI	cctc	6	25 75169 204 274 295	4	25 75 204 315
MseI	**t/taa**	**1**	**255**	-	-
MspR9I	*cc/ngg*	-	-	*1*	*266*
MvaI	*cc/wgg*	-	-	*1*	*266*
NdeII	**/gatc**	**1**	**188**	-	-
NlaIV	**ggn/ncc**	**2**	**49 190**	-	-
PalI	**gg/cc**	**1**	**270**	-	-
PflMI	**ccannnn/ntgg**	**1**	**44**	-	-
PleI	**gagtc**	**1**	**222**	-	-
Pme55I	**agg/cct**	**1**	**270**	-	-
PspN4I	**ggn/ncc**	**2**	**49 190**	-	-
PpuMI	*rg/gwccy*	-	-	*1*	*188*
Psp5II	*rg/gwccy*	-	-	*1*	*188*
RsaI	**gt/ac**	**1**	**279**	-	-
Sau3AI	**/gatc**	**1**	**188**	-	-
Sau96I	g/gncc	1	48	1	188
ScaI	**agt/act**	**1**	**279**	-	-
ScrFI	*cc/ngg*	-	-	*1*	*266*
SfaNI	*gcatc*	-	-	*1*	*46*
SinI	g/gwcc	1	48	1	188
Sse9I	/aatt	1	252	1	52
SseBI	**agg/cct**	**1**	**270**	-	-
StuI	**agg/cct**	**1**	**270**	-	-
TfiI	g/awtc	2	66 108	3	78 108 288
Tru1I	**t/taa**	**1**	**255**	-	-
Tru9I	**t/taa**	**1**	**255**	-	-
Tsp509I	/aatt	1	252	1	52
TspEI	/aatt	1	252	1	52
Van91I	**ccannnn/ntgg**	**1**	**44**	-	-
XhoII	**r/gatcy**	**1**	**188**	-	-

Restriction enzymes which cut the cytochrome b gene fragment at different sites in tiger and leopard are listed in Fig. 2.22 and Table 2.24. These can be used to differentiate tiger and leopard species from one another. Forty-two restriction enzymes are specific to tiger DNA fragments and cuts at various positions marked in bold in Table 2.24. Fourteen restriction enzymes were specific to leopard DNA sequences shown as italics in Table 2.24. Twenty-two restriction enzymes cuts both tiger and leopard sequence but, at variable positions.

Table 2.25. Restriction enzymes which are cutting partial fragment of 16S rRNA gene of tiger and leopard at different sites.

Enzyme name	Recognition sequence	*Panthera tigris*		*Panthera pardus*	
		No. of cuts	Positions of sites	No. of cuts	Positions of sites
AclWI	ggatc	1	287	2	58 287
Acc113I	*agt/act*	-	-	*1*	*267*
AfaI	*gt/ac*	-	-	*1*	*267*
Alw26I	gtctc	2	45 146	1	45
AlwI	ggatc	1	287	2	58 287
BglII	*a/gatct*	-	-	*1*	*143*
BsaI	**ggtctc**	**1**	**147**	-	-
BsmAI	gtctc	2	45 146	1	45
BsmFI	gggac	1	10	2	10 169
Bsp143I	/gatc	2	282 295	4	53 143 282 295
Bsp19I	**c/catgg**	**1**	**182**	-	-
BssT1I	c/cwwgg	2	182 315	1	315
BstX2I	*r/gatcy*	-	-	*1*	*143*
BstYI	*r/gatcy*	-	-	*1*	*143*
Csp6I	*g/tac*	-	-	*1*	*266*
DpnI	ga/tc	2	284 297	4	55 145 284 297
DpnII	/gatc	2	282 295	4	53 143 282 295
Eco130I	c/cwwgg	2	182 315	1	315
Eco31I	**ggtctc**	**1**	**147**	-	-
EcoT14I	c/cwwgg	2	182 315	1	315
Eco255I	*agt/act*	-	-	*1*	*267*
ErhI	c/cwwgg	2	182 315	1	315
Hsp92II	**catg/ g/cgc**	**1**	**186**	-	-
Kzo9I	/gatc	2	282 295	4	53 143 282 295
MboI	/gatc	2	282 295	4	53 143 282 295
MflI	**r/gatcy**	**1**	**143**	-	-

MseI	t/taa	3	124 128 241	4	124 128 148 241
NcoI	**c/catgg**	**1**	**182**	-	-
NdeII	/gatc	2	282 295	4	53 143 282 295
NlaIII	**catg/**	**1**	**186**	-	-
RsaI	*gt/ac*	-	-	*1*	*267*
Sau3AI	/gatc	2	282 295	4	53 143 282 295
ScaI	*agt/act*	-	-	*1*	*267*
StyI	c/cwwgg	2	182 315	1	315
Tru1I	t/taa	3	124 128 241	4	124 128 148 241
Tru9I	t/taa	3	124 128 241	4	124 128 148 241
XhoII	*r/gatcy*	-	-	*1*	*143*

Restriction enzymes which cuts the 16S rRNA gene fragment at different sites in tiger and leopard are listed in Fig. 2.23 and Table 2.25. These can be used to differentiate tiger and leopard species from one another. Seven restriction enzymes were specific to tiger DNA fragments shown in bold letters in Table 2.25. Ten restriction enzymes only cut leopard DNA sequences at various positions shown in italics in Table 2.25. Twenty restriction enzymes cuts both tiger and leopard sequence but, at different positions.

Table 2.26. Primers developed for tiger and leopard DNA using Primer 3 software of cytochrome b region.

Primer		Fragment size	Tm	GC contents
Forward	**Reverse**			
ACCCTGACACGA TTCTTTGC	CCCAGGATGTC TTTGATTGTG	173	60.24	48.81
ACACGATTCTTT GCCTTTCG	CCCAGGATGTC TTTGATTGTG	167	60.31	46.31
CCACCCTGACAC GATTCTTT	GACCCAGGATG TCTTTGATTG	177	59.69	48.81
ACCCTGACACGA TTCTTTGC	GACCCAGGATG TCTTTGATTG	175	59.76	48.81

Table 2.27. Primers developed for tiger and leopard DNA using Primer 3 software of 16S rRNA region.

Primer		Fragment size	Tm	GC contents
Forward	Reverse			
GTGAAGAGGCGG GAATATGA	GGATTGCGCTG TTATCCCTA	261	60.05	50
GAAGAGGCGGG AATATGACA	GGATTGCGCTG TTATCCCTA	259	60.05	50
CCAATCCGTGAA ATTGACCT	GGATTGCGCTG TTATCCCTA	285	59.93	47.5
AAGAGGCGGGA ATATGACAA	GGATTGCGCTG TTATCCCTA	258	59.93	47.5

These primers were designed for tiger and leopard DNA using Primer 3 software version 4, to amplify smaller segments of DNA, as the yield of DNA from bones were of shorter in fragment size (Table 2.26 and 2.27). These are universal primer and can be used to amplify both tiger and leopard DNA template. After amplification species can be determined through DNA sequences.

2.6. Discussion

Tiger (*Panthera tigris*) and leopard (*Panthera pardus*) bones are used as an ingredient for Traditional Chinese medicine and these animals are poached for their various parts. Thus, to identify these bones it is important to know its morphology (2.1 - 2.4 and Table 2.2 and 2.6) and use measurements to distinguish tiger and leopard bones. The skeletons of all cat species are very similar and their identification must be accurate if it is kept for comparative purpose. Even the skull fragments are often hard to distinguish, but if observed carefully all have some or the other differences (Lawrence 1968).

Five variables viz. maximum zygomatic width (MZW), facial length (FL), total skull length (TSL), crown length of canine (CL C1) and nasal length (NL) played major role in differentiating tiger skull from leopard (Fig. 2.5 and Table 2.9). Use of Discriminant function analysis for scapula and pelvic girdle for tiger and leopard indicates that these bones could be differentiated between tiger and leopard. First function itself could explain 100% variability (Table 2.10). Four variables were used to derive a function (Table2.11), height of scapula (HS), greatest dorsal length (LD), smallest length of the neck (SLC), and greatest length of scapula spine (GLSS) (Fig. 2.1). Pelvic bone of tiger and leopard could also be differentiated using Discriminant function analysis absolutely. First function itself could explain 100% variability (Table 2.14). Only one variable was used to derive a function that was greatest width across the tuber ischiae of pelvic (GBTI) (Fig. 2.2 and Table2.15). Three dimensional plots using variables, greatest length, inter-condylar distance and smallest width at diaphysis could separate femur of tiger and leopard. These variables can be used to differentiate femur of tiger from the leopard (Fig. 2.6). Even humerus of tiger and leopard could be distinguished using, greatest length, width of trochlea and smallest width of diaphysis (Fig. 2.7). Thus, these measurements could be efficiently used to distinguish major bones of tiger and leopard bones.

Ubelaker *et al.* (2002) could distinguish dental and osseous tissue using SEM/EDX, although it could not differentiate between ivory, mineral apatite and some corals. Scanning electron microscopy reveals best distinction at the core portion of tiger and leopard bones as there were numerous oblong holes (length 5000 μm and width 3000 μm) in tiger bone presently only one hole could be visible in the micrograph (Fig. 2.10) where as in leopard bone, the hole was smaller and not oblong (length 1000 μm and width 1500 μm). Few differences were also observed at other two portions viz. outer and cortex. Surface topographies identified at different portions using scanning electron microscope shows that pieces of tiger and leopard bones can be identified using this technique. At least exclusion principle may work that the unknown sample belongs to tiger or leopard.

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XRD analysis of tiger and leopard bones indicate presence of two shoulder peaks in leopard diffractogram at 25 and 26 ^{0} but, it was not consistent in all the diffractogram. Few peaks were also noticed between 42-44^0 angles in leopard bones diffractogram, where as these were absent in tiger diffractogram. However no clear demarcation was found in the diffractograms of tiger and leopard (Fig. 2.11). More data interpretation is needed for distinction between tiger and leopard bone through X-ray diffraction.

Thermo gravimetric analysis was successful in differentiating tiger and leopard bones. The two peaks in tiger and leopard bones indicate different rate of reaction at approximately similar temperature. The rate of loss was 0.7%/min at 56 ^{0} C at first peak in leopard compared to 1.2%/min rate of loss at 58 ^{0} C in tiger. The rate of loss in leopard was 1.9%/min at 328 ^{0} C at the second peak, where as tiger had rate of weight loss of 3.8%/min at 329 ^{0} C, that was twice than leopard. There was only one peak of energy loss of 767 mj/mg at 335 ^{0} C in leopard but, in tiger there were two peaks of energy loss of 836 mj/mg at 335 ^{0} C and 180 mj/mg at 454 ^{0}C (Fig. 2.12). The nature of weight loss pattern was similar but it was always low in leopard as compared to tiger (Fig. 2.13). The water loss percentage was approximately 8% in tiger where as in leopard it was 5%. There was difference in weight left at 1400 ^{0} C and it was 70.30% in leopard and 56.55% in tiger (Fig. 2.14). Thus, there were many differences which can be used even to determine from powder of bones that it belongs to tiger or leopard.

The elemental analysis includes screening through XRF intensities of tiger and leopard bones and further quantification through inductively coupled plasma – mass spectrometry. XRF indicate that there were differences in mean intensity of sodium, aluminum, silicon, chlorine and iron. These were high in tiger than leopard except iron. Thus, intensity of chlorine, sodium, aluminum and iron can be used to distinguish tiger and leopard bones (Fig. 2.15). ICP-MS analysis indicates relatively higher mean concentrations of calcium (Ca), barium (Ba), cobalt (Co) and strontium (Sr) in leopard bone as compared to tiger. Iron (Fe), magnesium (Mg), sodium (Na), lead (Pb), nickel (Ni), copper (Cu), and chromium (Cr) mean concentrations were detected more in tiger

bone as compared to leopard (Fig. 2.16). Major differences detected in bones of tiger and leopard were of Ca, Cu, Cr, Ba, Sr, Fe, Mg and Na and these may be used in differentiating the bones of these two species. Discriminant function analysis was developed in order to distinguish tiger bones from leopard using elemental composition. Discriminant function analysis indicates that a function could differentiate the tiger and leopard bones absolutely. First function itself could explain 100% variability (Table 2.20). Three variables were used to derive a function (Table2.21). The variables used in the function were as follows: calcium (Ca), copper (Cu) and sodium (Na) concentrations. Classification accuracy using one function for bones of tiger and leopard was found to be 100%.

Although DNA analysis is a highly discriminatory method, it is not self-sufficient and could not replace an anthropological evaluation (Budimlija *et al.* 2003). Hochmeister *et al.* (1991) amplified and typed DNA extracted from compact bone of human remains and could successfully use in establishing the identity of a person, as well as in excluding possible false identifications. The possibility of DNA in skeletal remains has been of great interest as these remains are preserved for longer duration and sometime only source of information for analysis. Bone DNA is present in minute amount trapped in calcium matrix hence difficult to extract DNA and there are also inhibitors of Taq polymerase. The success lies on proper method of extraction and removal of inhibitors for amplification of DNA. Though, it is difficult to extract DNA from hard tissues but, fairly goods amount of DNA (300-400bp) were extracted from bone of tiger and leopard using Gene clean and Bio Robotic methods (Fig.2.17).

PCR-based typing of short tandem repeats (STR) is routinely used as an established method for forensic practice. Frequently, however, STR typing fails in samples of poor quality including highly degraded specimens, old bone fragments or hair shafts without roots. For these types of samples, mitochondrial DNA (mtDNA) analysis has been successfully introduced (Corach *et al.* 1997; Seo *et al.* 2000). Polymerase chain reaction (PCR)-based DNA typing methods combined with standard anthropological approaches now play an important role in bone identification (Imaizumi *et al.* 2002;

Cattaneo *et al.* 1999). PCR of DNA samples extracted from bone of tiger and leopard were amplified successfully (Fig. 2.18). PCR was also done with 16S rRNA primer. Sequence was improved by cleaning the PCR product. These sequences were matched with NCBI data and checked for the species (Fig. 2.19). Analysis of alignment of tiger and leopard sequences indicates presence of adequate polymorphism to distinguish these two species. Analysis of sequences in Fig. 2.20 revealed more then 20% polymorphic sites in cytochrome b region (69 different sites were observed out of 319bp) and Fig. 2.21 shows that 5.52% sites were polymorphic for 16srRNA region (20 different sites out of 362 bp).

Restriction enzymes which were cutting the cytochrome b gene fragment at different sites in tiger and leopard were listed in Fig. 2.22 and Table 2.24. Forty-two restriction enzymes only cuts tiger DNA fragments at various positions and some of the enzymes are AccB71, AclWI, AfaI, Alw26I, AlwI. Fourteen restriction enzymes only cuts leopard DNA sequences at various positions and some useful enzymes are ScrFI, and SfaNI. Twenty-two restriction enzymes cuts both tiger and leopard sequence but, at variable positions e.g. AluI, TfiI. Thus, these restriction enzymes can be used to differentiate tiger and leopard species. Restriction enzymes which were cutting the 16S rRNA gene fragment at different sites in tiger and leopard were listed in Fig. 2.23 and Table 2.25 and may be used to differentiate tiger and leopard species. Seven and ten restriction enzymes only cuts tiger DNA fragments e.g. NlaIII, MflI and leopard DNA sequences e.g. XhoII, RsaI respectively and twenty restriction enzymes cuts both tiger and leopard sequences but, at variable positions e.g. AclWI, Tru9I.

Primers for both tiger and leopard of smaller fragment length were developed 167-177 bp for cytochrome b (Table 2.26) and 258-285 bp for 16S rRNA gene (Table 2.27) to amplify smaller fragments which were extracted from bones. After amplification with these primers, sequencing can be accomplished to differentiate tiger and leopard bones.

This chapter clearly indicates that bones of tiger and leopard may be identified using morphometry, SEM, x-ray fluorescence, inductively coupled plasma - mass spectrometry, thermo gravimetric and DNA analysis. Morphometry could differentiate the bones of tiger and leopard successfully in complete form. Few differences were observed in scanning electron micrographs, of tiger and leopard femur bones at core portion which is suitable to differentiate these two species. Not much distinct differences could be interpreted from X-ray diffraction pattern of bones of these two species. Therefore, the hypothesis that morphological and DNA characteristics of bones of tiger and leopards are species specific may be accepted but, the hypothesis that crystallography (XRD and XRF) are species specific for bones of tiger and leopard may be partially accepted. Thermo gravimetry of tiger and leopard bones has their species specific characteristics.

Based on the above study grading of different methods can be done to characterize the bone samples. While doing this, accuracy of the methods, their cost effectiveness, form of the samples, technical limitations, duration of analysis and utility of the methods has been kept in mind. It can be concluded that if bone samples are in complete form, morphometry is the best technique to characterize the bones and it is also cost effective. Overall TGA was found second most effective for all kind of samples. Though, ICP-MS and DNA are reliable and advanced techniques, getting DNA isolated from bone samples is difficult.

Table 2.28. Ranking order of different techniques for identifying species from bones of tiger and leopard.

Types of samples	Grading to use different techniques						
	Morphometry	SEM	XRD	TGA	XRF	ICP-MS	DNA
Complete	I	V	VII	II	VI	III	IV
Pieces	-	III	VI	I	V	II	IV
Powder	-	-	V	I	VI	II	III

Ranking is in decreasing order: I = Best

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CHAPTER 3

Characterization of rhinoceros horn

3.1. Introduction

There are five species of rhinoceros in the world, of which two, black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratothorium simum*) are found in Africa and rest three viz. Sumatran rhinoceros (*Didermocerus sumatrensis*), Javan rhinoceros (*Rhinoceros sondaicus*) and Indian rhinoceros (*Rhinoceros unicornis*) are found in Asia (Dinerstein 2003). Both African species and one of the Asian species (*Didermocerus sumatrensis*) has two horns, instead there is only one horn in other two Asian species (Dinerstein 2003). All five species of rhinoceros are endangered (Dinerstein 2003) and are placed in Appendix - I of the CITES. The total population of Asian species is fewer than 2,700 (Foose and Van Strien 1997). The Indian rhinoceros which presently survives in few pockets of India, Bhutan and Nepal is represented by approximately 2000 individuals (Khan and Foose 1994; West 1996; Dinerstein 2003). India has approximately 1500 Indian rhinoceros (Hanfee 1998), confined to few areas of Assam (Kaziranga, Orang, Loakhawa & Pobitora), W. Bengal (Jaldapara & Gorumara) and Uttar Pradesh (Dudhwa) (Ali *et al.* 1999).

The rhinoceros populations in Africa and Asia have been badly affected due to reckless poaching (Leader-Williams 1992; Amin *et al.* 2003) and extensive loss of habitat (Dinerstein 2003). The prime cause of poaching is illegal trade of rhinoceros horns for traditional uses in medicines (Dinerstein 2003), sculptors (Hsieh *et al.* 2003) and dagger handles (Emslie and Brooks 1999). Rhinoceros horn is used for fever which does not have any other cure thus highly prized and according to popular belief, Asian cultures value rhinoceros horn as a powerful aphrodisiac (Dinerstein, 2003). Rhinoceros horn, have been used as medicine in China, Burma, Thailand and Nepal in the treatment of haemorrhoids, arthritis, lumbago and polio (Penny, 1987). The gravity of this problem has been elaborated by various studies undertaken in Asia and Africa. Martin *et al.* (1997) reported that at least 67,000 kg of horn were imported to Yemen between 1970

and 1997. It clearly indicates that at least 22,350 rhinoceros would have been poached in Africa during the above indicated period to meet this demand (Dinerstein 2003). During 1960s, 70s, and 80s, a catastrophic poaching wave has wiped out 96% population of Black rhinoceros (Sherriffs 2005). The number of Northern white rhino has also declined drastically due to poaching from about 2,230 in 1960 to 15 by 1984 (Amin *et al.* 2003). In India, Menon (1996) reported poaching of 692 Great Indian one horn rhinoceros between 1980 and 1993. He further reports that 227 rhinoceros were poached alone in Kaziranga National Park of Assam between 1994 and 1996. In Jaldapara Wildlife Sanctuary, Assam, India, rhinoceros population declined from 76 in 1966-67 to 14 by 1980 due to poaching (Mukherjee and Sengupta 1998).

Steps have been taken to curb the reckless poaching. The international commercial trade of rhinoceros and their products was banned in 1976 under the CITES convention (Leader-Williams 1992). Consumer countries like China, South Korea, Taiwan and Hong Kong also banned the manufacture and trade in rhinoceros horn in the year 1993. To reduce rhino horn demand, its substitutes like horns of saiga antelope and water buffalo have also been tried (Penny 1987; Hay 1991; Dinerstein 2003). Despite, the legal protections and prohibitory measures, illegal trade of rhinoceros horn and its products still continues, due to high demand in international market. Absence of proper identification methods for rhinoceros horn while, in processed and powdered form (Penny 1987), is another problem for enforcement agencies to convict offender.

The rhinoceros horn does not have bony central core like horns of other ungulates, instead it is attached to the dermis covering the bone and are associated with bony rugosities (Hieronymus and Witmer 2004). Rhinoceros horn is epidermal derivatives composed of keratinized tubules of cells embedded in keratinized amorphous matrix. Ryder (1962) reported that the tubules comprise of ~ 40 lamellae of epithelial cells and range from 300-500 μm in diameter. According to Lynch *et al.* (1973) the amorphous matrix is made up of keratinized fusiform interstitial cells.

Characterization of the rhinoceros horn is very important to support legal intervention. Since the Asian horns are believed to be more efficacious and comparatively expensive than African horns (Nowell *et al.* 1992; Dinerstein 2003), the former are more prone for illegal trade.

Several studies have been undertaken to characterize rhinoceros horn of African species based on various techniques (Block 1939; Hsieh *et al.* 2003; Hoesch and Steven 2004; Hieronymus *et al.* 2006) as well as Asian species (Kingdon 1979; Ali *et al.* 1999; Amin *et al.* 2003; Dinerstein 2003). Kingdon (1979) reported that in greater one-horned rhinoceros, female often have longer horns than the males as later use horn more frequently for scraping. Dinerstein (2003) tried to classify rhinoceros horn into different age class using length and width of the horn. He successfully demonstrated the difference in the juvenile, sub-adult and adult horns.

Hieronymus *et al.* (2006) examined the nasal and frontal horns of the white rhinoceros using X-ray computed tomography (CT scanning), gross observation of sectioned horn, and light microscopy of histological sections of the horn tissue. He observed that horn is deposited dorsoventrally in successive sheets (horn lamina) with irregular layers of ~ 1-2 mm in nasal and ~ 0.5 -2.0 mm in frontal horn. In transverse section, horn laminae appear as bands. Under UV light the central part of each lamina is darker in colour than the periphery. The central dark patch is not uniform along length of the horn, rather these darker horn are interspersed with lighter horn at an interval of ~ 6 cm in nasal and 2 cm in frontal horns. Hieronymus *et al.* (2006) further reveal that these differences in contrast in radiography can be attributed to higher concentrations of calcium salts accompanying melanin deposition in the dark patches. The distribution of these two components in the horn contributes to the differential wear pattern and maintains the characteristic conical shape of the horn.

Several authors (Lee-Thorp *et al.* 1992; Hall-Martin *et al.* 1993; Hart *et al.* 1994; Emslie *et al.* 2001) have used chemical techniques to differentiate species and geographical source of the African rhinoceros horn, based on the variation in the concentration of elements and isotopes and their ratios. Amin *et al.* (2003) used three different chemical techniques; Mass Spectrometry for carbon and nitrogen isotopes, Inductively coupled plasma-optical emission spectrometry (ICP-OES) to know concentrations of four major and sixteen trace elements and Laser ablation - Inductively coupled plasma - Mass spectrometry (LA-ICP-MS) to measure the relative abundance of 132 isotopes of 58 elements to analyze the rhinoceros horns. He compiled a database of their chemical characteristics using the above three techniques and used Discriminant Function Analysis (DFA) to distinguish between species and origin of illegally traded or confiscated African rhinoceros horn.

Block (1939) investigated comparative biochemical composition of amino acids of keratins (eukeratins) of hair, wool, horn, nails, quills, feathers, skin and egg shell membrane. A relative consistency was noticed in the ratio of histidine, lysine and arginine which was found 1:4:12 or 1:5:12 in all cases, except goat hair. The amount of cystine was comparatively low in rhinoceros horn, goat hair, cattle horn, porcupine quills and hen feathers. But the tyrosine component was found highest (app. 9%) in rhinoceros horn and echidna spines as these are hard substance.

Molecular studies of rhinoceros tissue, horn and blood, mostly deals with phylogenetic issues (Ashley *et al.* 1990; Swart *et al.* 1994; Tougard *et al.* 2001). Few authors (Ali *et al.* 1999; Hoesch and Steven 2004; Hsieh et al. 2003) have used molecular techniques to identify rhino horn. Ashley *et al.* (1990) analyzed the mitochondrial DNA (mt DNA) of black rhinoceros and reported a low level of intra-specific genetic variation. Oryan *et al.* (1993) used cell culture and created map through comparative analysis of mt DNA to show variation among black rhinoceros sub-populations, but however no critical differences could be established. Swart *et al.* (1994) through their allozyme study in African and Asian rhinoceros revealed low level of intra specific variation. Swart and

Ferguson (1997) examined the genetic characteristic of South African populations of black rhinoceros, and revealed statistically significant differences between populations. Tougard *et al.* (2001) analyze phylogeny through the complete sequences of the mitochondrial 12 S rRNA and cytochrome b genes, and estimated that the African and Asian lineages have diverged at about 26 million years before and identified the Sumatran rhinoceros as a sister group of rhinoceros. Hsieh *et al.* (2003) sequenced a 402 bp fragment of cytochrome b, to successfully identify horn of white, black and Indian rhinoceros. Wurster and Benirschke (1968) reports that *Rhinoceros unicornis* has 82 chromosomes, with male being heterogametic and female being homogametic. Ali *et al.* (1999) cloned and sequenced a 906 bp EcoRI repeat DNA fraction from *Rhinoceros unicornis* genome. He revealed that the AT rich contig pSS(R)2 sequences are unique to *Rhinoceros unicornis* genome because they do not cross-hybridize, even with the genomic DNA of South African black rhino *Diceros bicornis* and can be potentially used for identification of horn or other body tissues of *Rhinoceros unicornis*. Hoesch and Steven (2004) developed a method to distinguishing between the African rhinoceros horns and those from various domestic and wild bovid species through extraction of DNA remaining in horn, amplification and direct sequencing of a portion of the mitochondrial Cytochrome *b* gene and comparing the sequence with a database of representative rhinoceros and bovid species.

The above literature review indicates that informative scientific studies have been done for identification of rhinoceros horn but, most of them are on African species. Almost all scientific studies are based on one or more than one techniques. It was intended to analyze the horn of Asian species and use most of the recent techniques available so, that wide spectrum of various forms of samples of rhinoceros horn can be identified. Also it will help in differentiating it from fake horns which are common in trade. Therefore, an attempt was undertaken for characterization of the Asian rhinoceros horn using morphometric, analytical and molecular techniques. Attempts were also made to develop species specific characteristics of Asian rhinoceros horn (*Rhinoceros unicornis*) to differentiate them from horn of other species. This will help in proper identification of the object which otherwise is given benefit of doubt in absence of

diagnostic identification. This would expect to reduce illegal trade of rhinoceros horn and will support in conservation of the endangered species.

3.2. Objective

Determine morphological, crystallographic (XRD & XRF) and DNA characteristics of greater Indian one horned rhinoceros.

3.3. Hypothesis

Morphological, crystallographic (XRD & XRF) and DNA characteristics of greater Indian one horned rhinoceros horn are species specific.

3.4. Materials and methods

All rhinoceros horns were procured from Forest Departments viz. Assam, West Bengal and Uttar Pradesh. Based on the work so far undertaken for characterization of rhinoceros horn all over the world, the Greater one horned rhinoceros horn was characterized using structural patterns, chemical constituents, protein profile and DNA based technique. Table 3.1 indicates about the methods used and number of samples analyzed.

Table 3.1. Different techniques and number of sample used to characterize rhinoceros and other horns.

Sl. No.	Techniques	Number of samples			
		Rhino horn from wild	Rhino horn from zoo	Fake rhino horn	Buffalo horn
1	Morphometry	6	2	2	-
2	Scanning electron micrography	3	2	3	1
3	X-ray diffraction	4	1	2	1
4	X-ray fluorescence	1	-	-	-
5	Inductively coupled plasma- mass spectrometry	2	2	-	-
6	Thermo gravitimetric analysis	1	2	-	-
7	Protein profile (SDS-PAGE)	3	2	1	1
8	DNA based	2	1	-	1

3.4.1. Morphometry of rhinoceros horn

For standardizing method to identify rhinoceros horn through morphometric measurements and morphological characteristics, total eight rhinoceros horns (six from wild, and two from zoo) were used. The rhinoceros horns from wild were of different sizes. Two fake rhinoceros horns were also included for morphometric characterization as fake rhinoceros horn made up of materials like buffalo horn, bone, wood, resin and plastics are also prevalent in illegal trade.

Twenty-three quantitative and nine qualitative variables related to morphological features were recorded. The 9 measurements related to qualitative variables of rhinoceros horn and 23 quantitative variables reveal about the morphological structure and its measurements respectively. The variables are given in the Table 3.2. All quantitative measurements were mainly recorded in millimeter (mm) using vernier caliper and where it was not possible to measure using vernier caliper it was measured with the help of thread and scale.

Colour of the horns was recorded visually. Horns were weighted using balance (P16, Mettler, Switzerland) in gm. Presence of dorsal hair and tubercles were noted under low magnification using stereo microscope Lieca MZ 125, Germany. Few morphologically distinct features were noted during physical examination of the horns. Angle of rhinoceros horn and fake rhinoceros horn formed due to bending of stem were measured (Fig. 3.1a). Angles were drawn on sheet of paper and later, it was measured using a protector. Four rhinoceros horn samples of varied size ranging from small to big horns were used for angle measurements (Fig. 3.1b). Graphs were plotted to find range of basal circumference of rhinoceros horn. In addition to this, weight/circumference were plotted to minimize the biases due to size of the horns. Finally, Discriminant functional analysis of the quantitative measurements was performed to differentiate fake and real rhinoceros horn. Further, density of the rhinoceros horn was measured using water displacement method using specific gravity measuring kit, AD-1653 of the weighing balance (GR 200, A & D Company Limited, Japan) at room temperature.

Table 3.2. Different qualitative and quantitative measurements used for rhinoceros horns.

Sl. No.	Qualitative measurements	Quantitative measurements
1	Colour	Total length from base to tip (on bent side) (TLBS)
2	Weight	Total length from base to tip (on unbent side) (TLUBS)
3	Dorsal surface texture (DST)	Length till dorsal surface of base (bent side) (LDSBBS)
4	Ventral surface texture (STV)	Length till dorsal surface of base (unbent side) (LDSBUBS)
5	Presence of skin membrane on ventral surface (SMVS)	Thickness of basal portion at diagonal (a) (TBSa)
6	Tip texture	Thickness of basal portion at diagonal (b) (TBSb)
7	Presence of growing hairs on the basal dorsal surface (PGHBPDS)	Thickness of basal portion at diagonals (c) (TBSc)
8	Presence of hairs above the base (PGHAB)	Thickness of basal portion at four diagonal (d) (TBSd)
9	Presence of tubercles (PoT)	Diameter of long axis (ventral surface) (DLAVS)
10		Diameter of short axis (ventral surface) (DSAVS)
11		Width of long axis on dorsal surface (WLADS)
12		Width of short axis on dorsal surface (WSADS)
13		Circumference of base (ventral surface) (CBVS)
14		Circumference of base (dorsal surface) (CBDS)
15		Greatest circumference of the vertical portion (GCVP)
16		Circumference at where uniformity starts in vertical portion (CUVP)
17		Circumference at middle portion of vertical portion (CMPVP)
18		Circumference at bent portion (CBP)
19		Circumference at tip (CaT)
20		Length of outgrowth present at ventral portion (LOpVP)
21		Height of the outgrowth (HoO)
22		Height of the center (ventral portion) (HCVP)
23		Bending angle (BA)

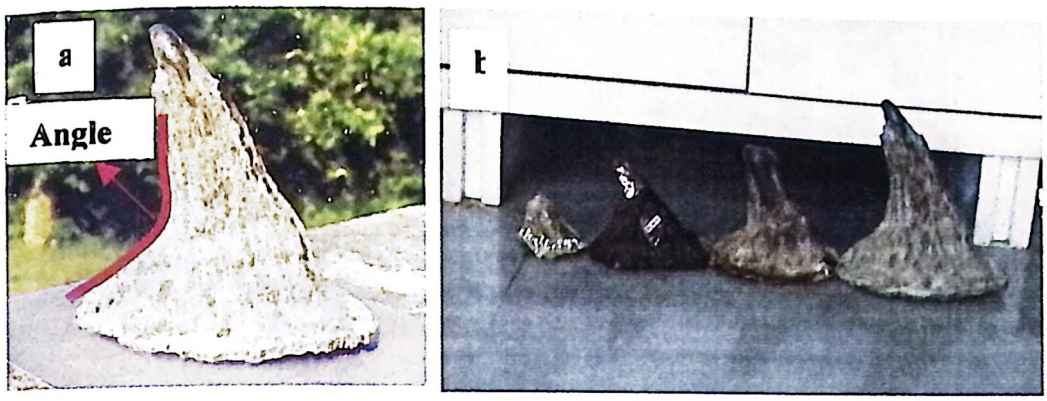


Figure 3.1. Different horns of rhinoceros used for morphometric studies. a. Measurement of angle; b. Horns of rhinoceros.

3.4.2. Scanning electron microscopy (SEM)

Total eight horns (three rhinoceros horns from wild, two from zoo and three fake rhinoceros horns) were examined under scanning electron microscope. Samples were prepared by cutting two small pieces of 5mm width, 5 mm length and 2 mm thickness, one from dorsal and other from ventral side from basal portion of rhinoceros horn and cleaned properly using alcohol. These small pieces were mounted on the aluminum stub, using silver paste and left for drying and were sputter coated with gold thickness (10-20 A^0) at 25 mA, 2.5 kV in argon atmosphere at 10-8 mbar/pa for 3 minutes using cool sputter coater (E5100 Series II, Polaron Equipment Ltd., U.K.) to facilitate their examination under the Scanning Microscope (PSEM 515, Philips, Holland). The acquired SEM micrographs were saved using image processor software, Digital Image Scanning System (DISS 5, Point Electronics, Germany). The micrographs obtained of the rhinoceros horns and fake rhinoceros horns were compared on both dorsal and ventral side, to note the species specific characteristics.

3.4.3. X-ray diffraction

Eight samples (four rhinoceros horn from wild, one from zoo, two fake rhinoceros horn and one buffalo horn) were subjected to X-ray diffraction analysis. The horn pieces were cut and crushed under ball mill to obtain 2-4 mg samples uniformly ground down to 300 mess size for analysis. Samples were tightly and homogenously packed on to a aluminum sample holder for acquiring diffraction pattern. Initially, X-Ray Diffractometer

model no. PW 1710, Philips, Holland at 55 mA current, 40 kV voltage and later on advanced version of Diffractometer PANalytical, Philips, Holland at 40 mA, and 45 kV were used for analyzing horns. For one of the rhinoceros horn complete scanning was done from 5^0 to 100^0 to know about the position of diffracted peaks. Later on scanning angle was restricted to 25 to 55^0 with a step size of 0.020 2θ and 5 sec counting time was used as optimum instrumental conditions. XRD diffraction pattern were analyzed and printed using XRD analysis software supplied by Philips, Holland and the X' Pert High Score plus from Pananalytical.

3.4.4. Thermo gravimetric analysis

Small pieces from three rhinoceros horn were cut, cleaned, further dried in desiccators and ground. Approximately 5 mg of powdered samples were analyzed under Perkin Elmer thermo analyzer using alumina powder as reference in nitrogen atmosphere 200 ml/min. Temperature was uniformly increased at the rate of 10 ^{0}C/min from 20^{0}C to 1400 ^{0}C. Thermograph and data were generated using Pyris Diamond software.

3.4.5. Elemental analysis

2.4.5.1. X-ray fluorescence

Pieces of a rhinoceros horn was cut and cleaned and uniformly ground to 200-300 mesh sized particles. Five gram of powdered sample was taken and mixed with one-two drop of poly vinyl alcohol and thoroughly homogenized. This mixture was placed under the pressing instrument and pressed under 2000 lbs/in^2 of pressure to prepare pellets. Pressure was released to take out the pellet from the holder. Pellet should be of 35-40 mm diameter and less than 10 mm thick. This pellet was desiccated overnight and qualitative and quantitative analysis of some important constituent elements found in rhinoceros horn was performed by Sequential X-ray fluorescence Spectrometer (Siemens SRS 3000, Varian, Australia). Due to lack of proper analytical standards, quantitative analysis by XRF is not performed. Instead only intensities (KCps) of fourteen elements were recorded to use it as semi-quantitative comparative purpose.

3.4.5.2 Inductively coupled plasma-mass spectrometry (ICP-MS)

Four rhinoceros horns (two from wild and two from zoo) were analyzed through ICP-MS. Samples were kept in cleaned and labeled teflon crucibles. Approximately 0.1 g of ground sample was measured and 10–15 ml of concentrated nitric acid and hydrofluoric acid in 1:2 ratios was added to the sample. These were boiled, until the sample dried. Same step was followed again and the sample was heated further. To above digested samples 5 ml of perchloric acid was added and boiled. Same step was followed and completely dried samples were extracted with 20% hydrochloric acid. The sample solution was made-up to 200 ml by adding distilled water and kept ready for instrumental analysis using Perkin-Elmer SCIEX ICP-Mass Spectrometer model ELAN DRC-e, USA. Instrumental concentrations of various elements were transformed into parts per million using standard formula.

3.4.6. Protein profile using sodium dodicyl sulphate poly accrylamide gel electrophoresis (SDS PAGE)

SDS PAGE of powdered samples (10 mg) of rhinoceros horn (n=5), buffalo horn (n=1) and fake horn (n=1) were digested for minimum 36 hours according to Butler *et al.* (1990) using 200 µL of a solution containing 12 M Urea, 74 M Trizma base, and 78 mM dithiothretol (DTT). Separating, spacer and stacking gels were prepared according to Schagger and von Jagow (1987); Judd (1996) inside the gel caster and the gel was left for drying.

A 25 µL aliquot of extract was removed and incubated with 5 µL of 0.1 M DTT for 10 min at 25^0C and extracted by adding sample solubilization buffer (2 ml 10% SDS in distilled water, 1.0 ml glycerol, 0.625 ml 1 M Tris-HCl, 6 ml distilled water, bromophenol blue to colour and pH should be 6.8) and then heating the digested sample to 96 ^{0}C. Sample was mixed with bromophenyl dye and 5-8 µl loaded for separation using standard protocols (Schagger and von Jagow, 1987; Judd, 1996) based on molecular weight using vertical Hoefer SE 260. SDS low molecular weight standard was run along with these samples for calibration of molecular weight. Finally the gel was

stained using coomassie dye and de-stained using de-staining solution and the bands were observed. The gel was photographed under UV light using UVP Bio-imaging System, Cambridge, U.K. Further analysis of these bands on the gel was done using Gene Profiler, version 4.5 software and molecular weight of the bands was determined with respect to the standard.

3.4.7. DNA analysis

Basal portion of rhinoceros horn was cleaned using alcohol or sodium hypochlorite (NaOCl) (Kemp and Smith 2005) and scraped to collect minimum 25 mg powder, for one time extraction of DNA. The sample was digested overnight at 55^0C using protocol of DNeasy Tissue Kit (QIAGEN, Germany) for animal tissue with slight modification of adding 0.39 mM DTT along with 180 µl ATL. Further, procedure to extract DNA, using protocol of commercially available DNeasy Tissue Kit (QIAGEN, Germany) was followed. 4-10 µl extracted DNA was resolved on 0.8% gel, and photographed (UVP Bio-imaging System, Cambridge, U.K.) under UV light. Further, the DNA fragments was cut and subjected to cleaning following the protocol of Qiagen gel purification kit, Germany.

Since, the cytochrome b and 16S rRNA genes are located in the mitochondrial genome and their highly conserved nucleotide sequences are known to be species-specific. Cytochrome b gene is widely analyzed for species-specific identification, phylogenetic and forensic investigations (Kocher *et al.* 1992; Hsieh *et al.* 2001; Wan and Fang 2003). Large ribosomal 16S rRNA gene has been used widely for species identification, as well as for phylogenetic analysis (Carrera *et al.* 1999; Stubbs *et al.* 2000).Then, partial fragments of cytochrome b gene 450 bp (Verma *et al.* 2003) and 16S rRNA gene of 550bp (Mitchell *et al.* 1993) of mitochondrial DNA were chosen for species identification.

Primer A for partial fragment of cytochrome b gene:

Forward, 5'-TACCATGAGGACAAATATCATTCTG-3',
Reverse, 5'- CCTCCTAGTTTGTTAGGGATTGATCG-3'

Primer B for partial fragment of 16S rRNA gene:

Forward, 5'-CGCCTGTTTATCAAAAACAT-3'
Reverse, 5'-CTCCGGTTTGAACTCAGATC-3'

Above mentioned primers were used for PCR amplification. PCR amplification reaction was carried out in a volume of 50 µl containing 1x- PCR Buffer (50mM KCl, 10mM Tris HCl (pH 8.8), 0.08% Nomidet P40), 1.5mM $MgCl_2$, 0.5 mM dNTPs, 0.2 µM of each primer, 0.1mg/ml BSA, 1U Taq polymerase, and 50-100ηg of DNA template. The reaction mixture was initially heat denatured at 94^0C for 2 min followed by 30 cycles of denaturation at 94^0C for 1 min, annealing were similar for both primers at 51^0C for 1 min, and extension at 72^0 C for 1 min, with a final extension step at 72^0C for 10 min. 6 µl of amplification products were resolved on a 2% agarose gel in 1xTAE buffer, stained with ethidium bromide and photographed under UV. Later, amplified PCR products were purified using QIAquick® PCR Purification Kit (QIAGEN, Germany). For sequencing, sequencing PCR was set using 2 µl RR big dye, 1 µl 5X buffer, and PCR template. Amplified PCR products were purified with ethanol precipitation and the product was sequenced on ABI 3130 (Applied Biosystem, USA) DNA sequencer.

DNA sequences of cytochrome b gene of *Rhinoceros unicornis*, *Bubalus bubalis* and *Bos taurus* from NCBI data base were aligned using software, Bioedit version 5.0.9 to see the pattern of variability in data. Nucleotide polymorphism for the gene was noticed. The restriction enzyme digestion map was identified by software, Webcutter 2.0 for the gene (cytochrome b). Attempts were made to find the restriction enzymes specific to *Rhinoceros unicornis.* Then, universal primer for all the three species was designed for cytochrome b gene using software, Primer3, version 4.

Statistical analysis

Statistical analysis used for morphometry were Mann-Whitney U test for testing goodness of fit, mean and standard deviation for different parameters and Discriminant

Function Analysis was carried out for the quantitative variables of morphometric measurements.

3.5. Results

3.5.1. Morphometry of rhinoceros horn

To identify complete horn, morphometry is efficient scientific method which can be used at field level. It comprises of two parts morphological features and measurements.

3.5.1.1. Morphological features

Weight of the rhinoceros horns ranged from 100.30 to 375.50 gm and 504.00 to 1042.90 gm in young and adult rhinoceros respectively. Mean weight and standard deviation of young and adult rhinoceros horns was 214.47±143.46 and 849.38±236.87 respectively. Rhinoceros horns observed were mostly of grey colour except, a sample which was dark brownish. Some of the fake rhinoceros horns also were of similar colour of real rhinoceros horn. The dorsal surface of rhinoceros horn is rough due to presence of hairs (Fig. 3.2a) and there are numerous tubercles present on its basal portion, due to uneven growth of hairs (Fig. 3.2b&c). A distinct furrow was noticed at the anterior portion of the dorsal surface of rhinoceros horn (Fig. 3.2d) Tip of the rhinoceros horn was either shining or dull (Fig. 3.2e).

Ventral surface was concave (Fig. 3.3a) in shape and contained numerous pores (Fig. 3.3b). It generally had two axes, one long axis and other short axis. A distinct feature was also noted on the ventral surface that is the presence of outgrowth or bulge (Fig. 3.3c). Dermal layer may or may not present on the ventral surface (Fig. 3.3d).

The average angles with standard deviation in rhinoceros horn (n=4) measured was $131.50^0 \pm 13.03$. The angles ranged from 115-145^0. However, the angle observed in fake rhinoceros horn (n=2) was $140.67^0 \pm 4.95$ and it ranged from 145-152^0 (Table 3.3). Average basal circumference of rhinoceros horns on ventral surface (Fig. 3.4a) was of 430±150.88, and the average basal circumference on the dorsal surface was of 406.33±133.78. The average basal circumference of zoo rhinoceros horns at ventral surface and dorsal surface were 490±141.42 and 477.75±159.45 respectively. The average basal circumference of fake rhinoceros horns at ventral surface and dorsal surface were 340±0 and 387.50±3.54 respectively (Table 3.4). Further, the circumference/weight was calculated to minimize the size effect. The circumference/weight value of rhinoceros horns was found between 0.6-2.6 mm/g, and in zoo rhinoceros horns was 1.25-2.25 mm/g, while this value in fake rhinoceros horns was between 0.2 and 0.5 mm/g (Fig. 3.4b).

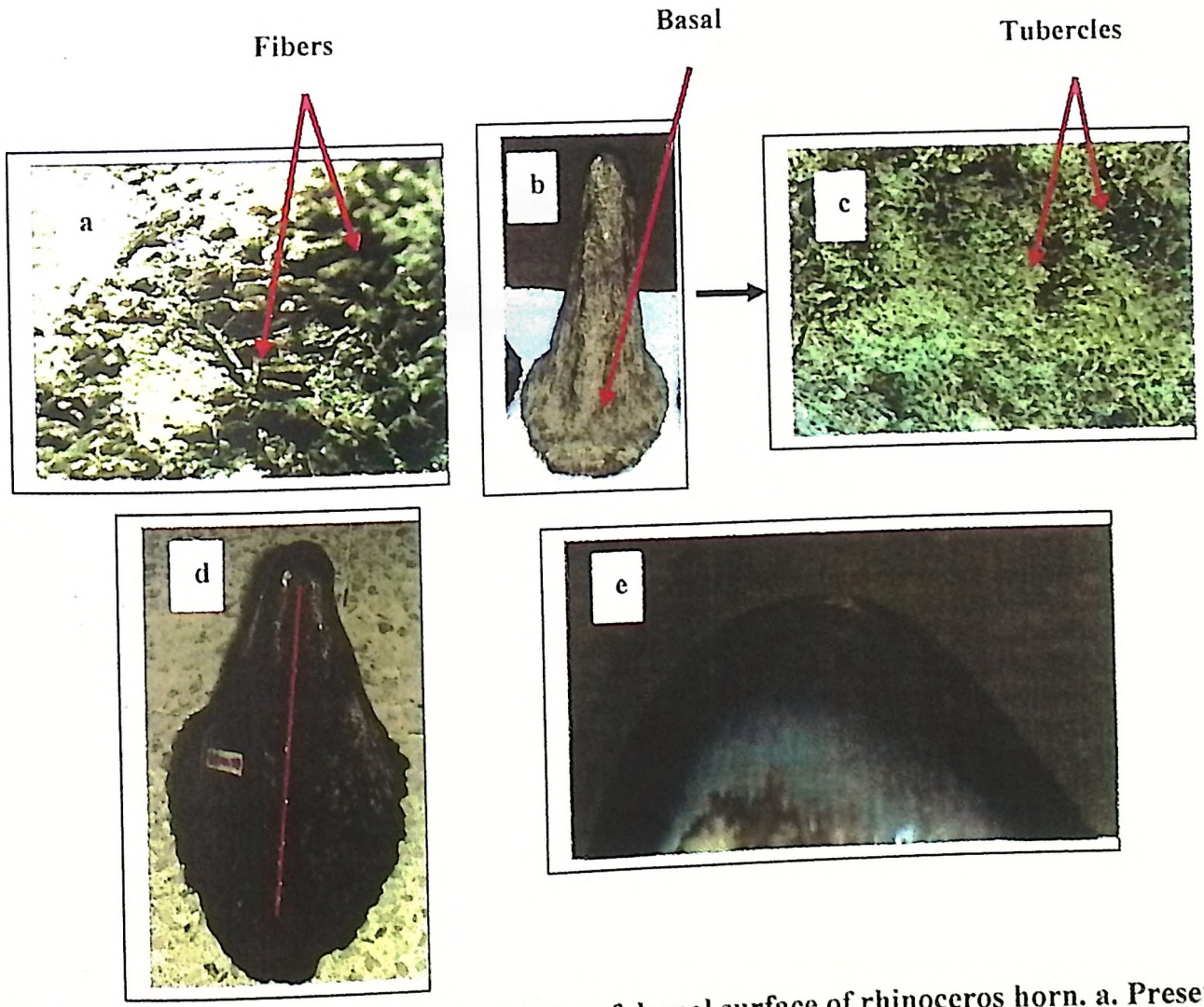


Figure 3.2. Morphometric characteristics of dorsal surface of rhinoceros horn. a. Presence of growing fibers under low magnification; b. Basal portion; c. Tubercles; d. Furrow; e.Tip.

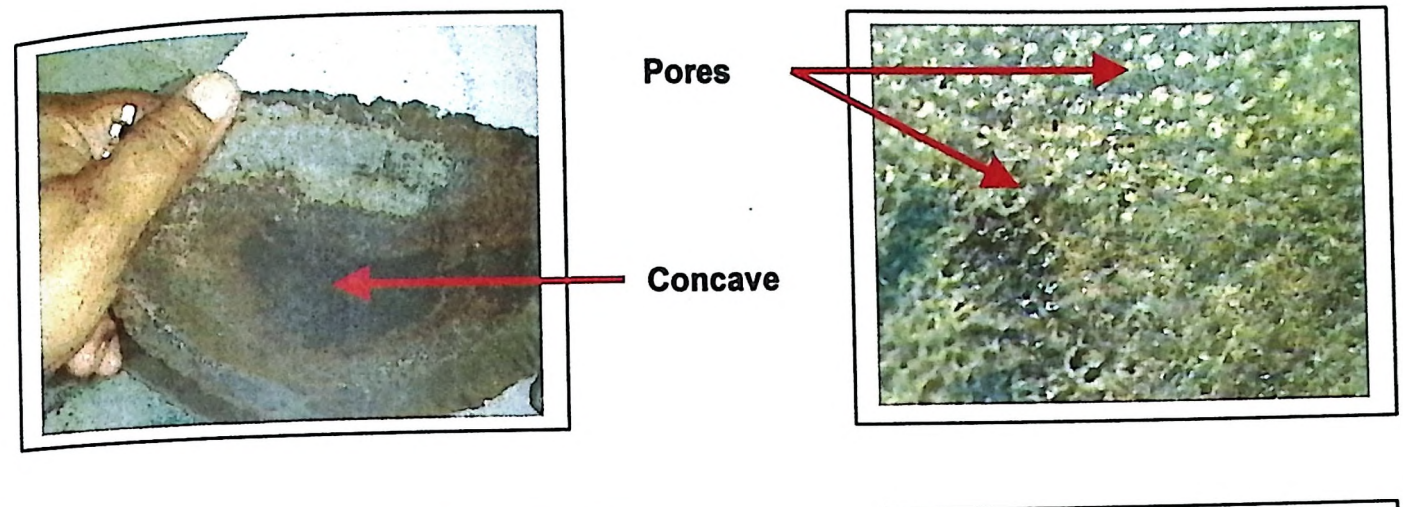


Figure3.3. Morphometric characteristics of ventral surface of rhinoceros horn. a. Concave surface ; b. Numerous pores; c. Outgrowth ; d. Presence of dermal layer.

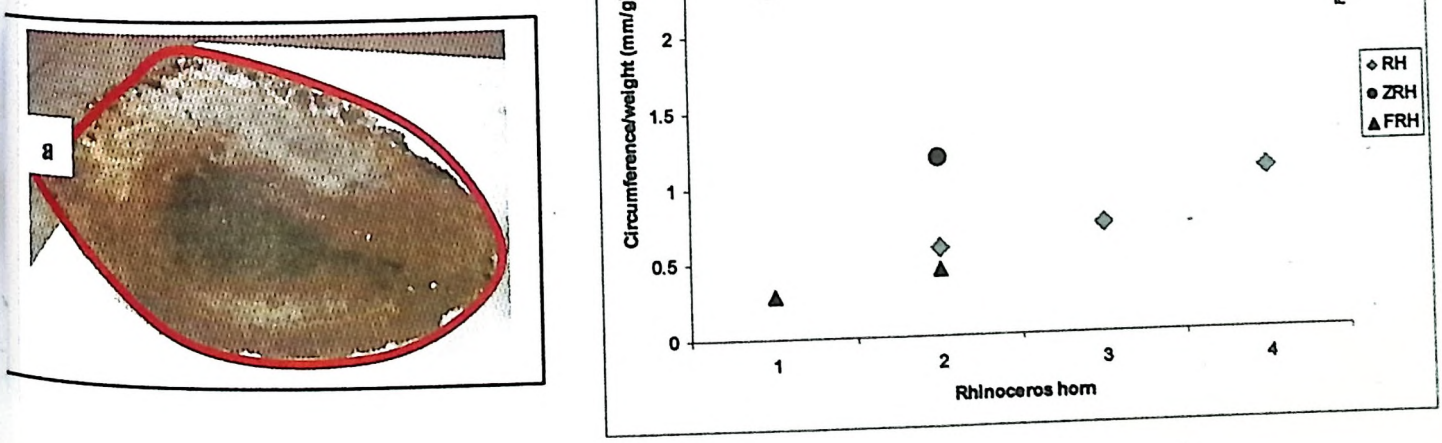


Figure 3.4. Differences based on circumference/weight of rhinoceros horn. a. Circumference of basal portion of rhinoceros horn; b. Plot of circumference/weight for rhinoceros horn from wild and zoo and fake.

Table 3.3. Angles (mean ± SD) observed in real and fake rhinoceros horns.

Variables	Rhinoceros horn	Fake Rhinoceros horn
No. of samples (N)	4	2
Average angle (^{0})	131.50 ±13.03	140.67 ± 4.95
Range	115-145	145-152

Table 3.4. Values (mean ± SD) of dorsal and ventral circumference of rhinoceros and fake horns.

Variables	RH	ZRH	FRH
N	6	2	2
Ventral circumference	430.83 ±150.88	490 ± 141.42	340 ± 0
Dorsal circumference	406.33 ±133.78	477.75 ± 159.45	387.50 ± 3.54

RH= Rhino horn; ZRH= Zoo rhino horn; FRH= Fake rhino horn

3.5.1.2. Statistical analysis

Missing values

In the datasets there were few missing values which were replaced by means through linear interpolation or wherever linear interpolation was not suitable series mean function was used to replace the missing values.

Discriminant Function Analysis (DFA)

Discriminant function analysis was developed in order to distinguish rhinoceros horn from fake horns. Discriminant function analysis was performed using stepwise method in which each of the variables was entered in a row and assessed for its significance of classification function using F statistic (i.e., F to remove 3.14). Since several of the variables would show multi-collinearity, Wilk's lambda values were used to ascertain the degree of collinearity and remove those variables from further analyses (Table 3.5).

Table 3.5.Variables used in the analysis.

Step	Variables	Tolerance	F to Remove	Wilks' Lambda
7	LINT(GCVP)	.01	74.03	.00
	SMEAN(LOPVP)	.01	59.13	.00
	LINT(TBSA)	.01	55.48	.00
	LINT(DSAVS)	.01	28.50	.00
	SMEAN(CMPVP)	.003	23.90	.00
	LINT(CUVP)	.01	10.25	.00
	LINT(LDSBUBS)	.04	6.49	.00

Two functions could differentiate the rhinoceros horn from wild and zoo as well as fake rhinoceros horn absolutely. First function itself could explain 96.9% variability (Table 3.6).

Table 3.6. Eigenvalues.

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	3733.195	96.9	96.9	1.000
2	119.107	3.1	100.0	.996

Standardized Canonical Discriminant Function Coefficients

Table 3.7. Standardized Canonical Discriminant Function Coefficients.

Variable	Function	Function
	1	2
LINT(LDSBUBS)	-2.64	4.01
LINT(TBSA)	8.66	3.98
LINT(DSAVS)	7.04	-9.50
LINT(GCVP)	-9.10	2.21
LINT(CUVP)	10.04	-6.44
SMEAN(CMPVP)	-15.12	7.69
SMEAN(LOPVP)	9.01	7.51

Seven variables were used to derive two functions (Table 3.7). The variables used in these two functions were as follows: greatest circumference of the vertical portion (GCVP), length of outgrowth present at ventral portion (LOPVP), thickness of basal portion at diagonal (a) (TBSA), length till dorsal surface of base (unbent side)

(LDSBUBS), diameter of short axis (ventral surface) (DSAVS), circumference where uniformity starts in vertical portion (CUVP), circumference at middle portion of vertical portion (CMPVP).

The discriminant functions for all the species are given in Table 4.8. The coefficients of these seven variables using Fisher's linear Discriminant functions were used to differentiate rhinoceros horn from wild, zoo and fake horn. The classification formula is: $D_i = C_i + (V_1 \times U_1) + (V_2 \times U_2) + (V_3 \times U_3) + \ldots\ldots + (V_n \times U_n)$.

where D_i = Discriminate score, C_i =Constant, V_1 = Value of the 1^{st} variable measured of the i^{th} sample and U_1 = Fisher linear coefficient value of the i^{th} sample for the i^{th} species. The unknown sample is assigned to the species for which the discriminant score is the highest. Classification accuracy for these samples was found to be 100%. Canonical Discriminant function was used to plot discriminant function 1 and discriminant function 2 (Fig. 3.5). The group centroids of rhinoceros horn from wild, zoo and fake horns formed distinct clusters.

Table 3.8. Fisher's linear Discriminant functions (Classification Function Coefficients).

Variable	RHINOHORN		
	Wild	Zoo	Fake
LINT(LDSBUBS)	1.87	-2.03	3.35
LINT(TBSA)	45.90	233.33	-148.13
LINT(DSAVS)	-7.66	10.18	-15.18
LINT(GCVP)	.971	-12.09	10.72
LINT(CUVP)	-3.65	12.97	-13.87
SMEAN(CMPVP)	3.97	-18.80	18.92
SMEAN(LOPVP)	25.76	82.34	-43.74
(Constant)	-705.12	-4249.34	-2704.42

Classification accuracy 100%

For parts of rhinoceros horn or sculptors made from rhinoceros horn or other horns sometime, it may not be possible to analyze through morphometry than we should use other techniques.

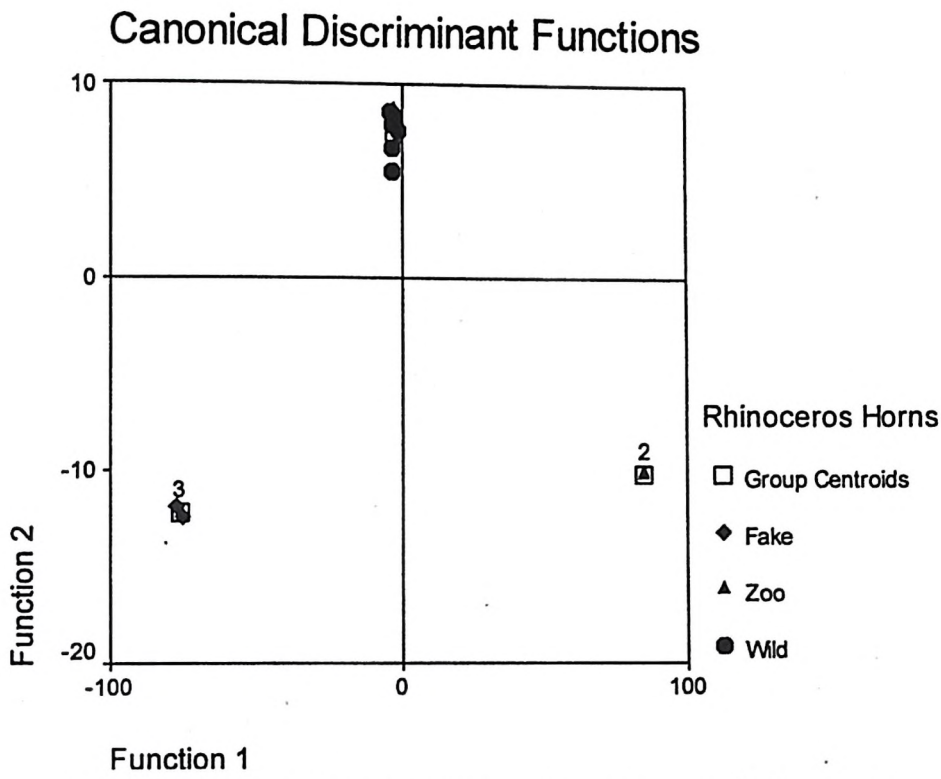


Figure 3.5. Canonical Discriminant function to differentiate real horn from zoo and fake rhinoceros horns.

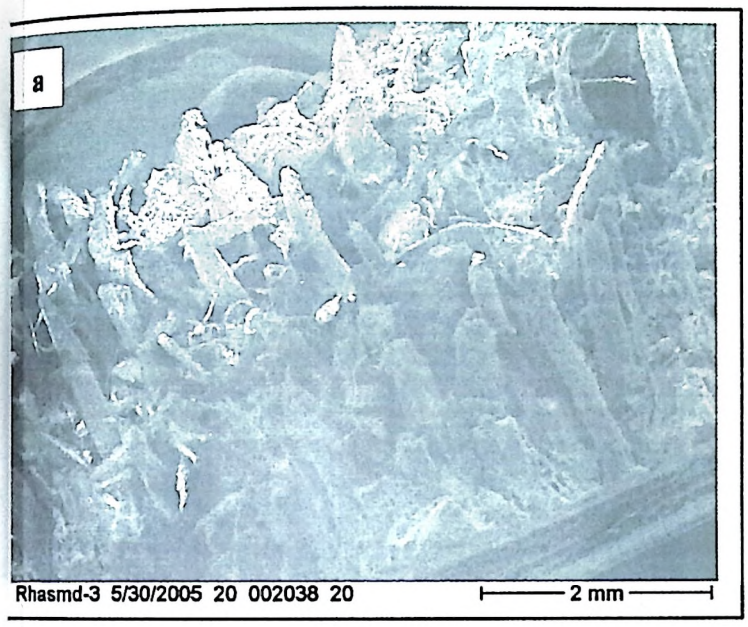
3.5.1.3. Density

Density of 1 cm^2 pieces of rhinoceros horn was 7.49 g/cm^3 and buffalo horn was 4.54 g/cm^3. Density is a good parameter to distinguish rhinoceros horn from buffalo horn (fake rhinoceros horn). Mann Whitney U test for densities were 0.0001 and Asymptotic significance (2-tailed) were 0.025.

3.5.2. Scanning electron microscopy

Both dorsal and ventral surfaces of rhinoceros horns were examined under scanning electron microscope. Differences were noted on both the surfaces. On the dorsal surface, hairs were observed growing compactly in real rhino horn (Fig. 3.6a), whereas there were no evidence of hairs in buffalo and fake horn (Fig. 3.6b&c), except in a sample were sparse hair growth were observed (Fig. 3.6d). On examination of ventral surface of rhinoceros horn numerous uniformly placed circular pores with mean diameter of 320 μm (range = 160-400 μm) were noticed (Fig. 3.7a). When a pore part was further magnified, many sub-pores of mean diameter of 9.39 μm (range = 5.56 to 10.11 μm) were visible within the pore (Fig. 3.7c). In case of fake horn, few ununiform pores were visible (Fig. 3.7b) and further magnification on a pore there were no sub-pores but, rough

surface was visible (Fig. 3.7d). The ventral portion of buffalo horn does not indicate presence of any pores (Fig. 3.7e). Its further magnification reveals presence of twisted fibers and had dark zone in the middle of these fibers (Fig. 3.7f).



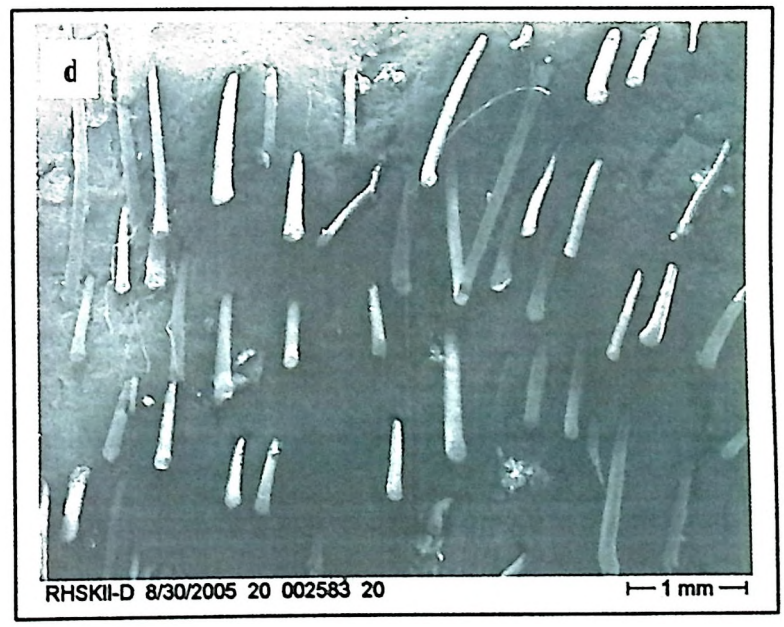
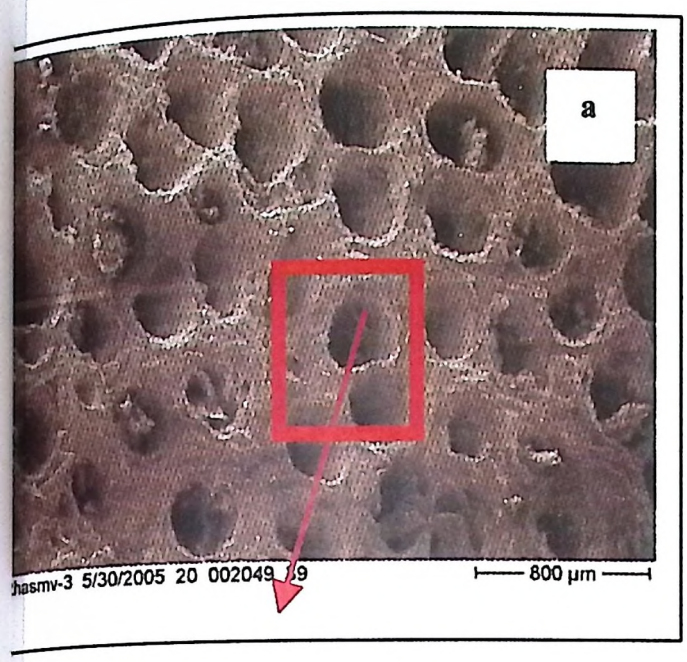
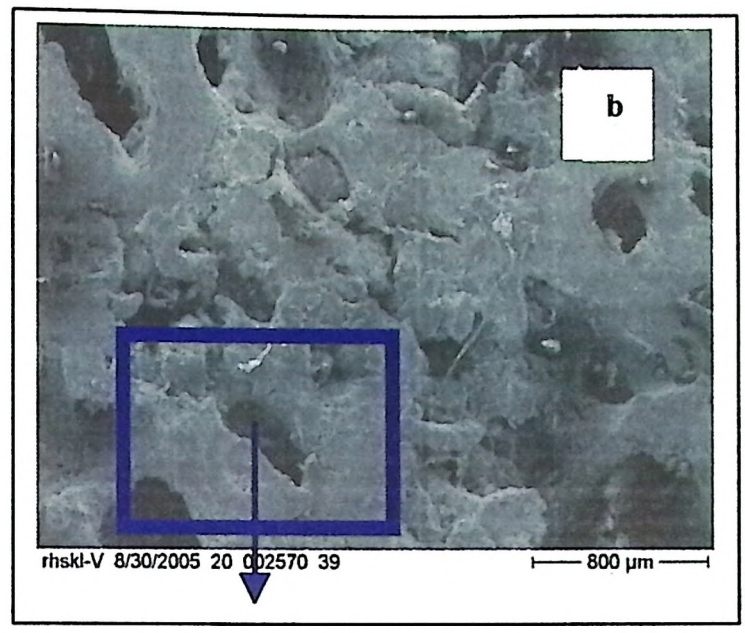
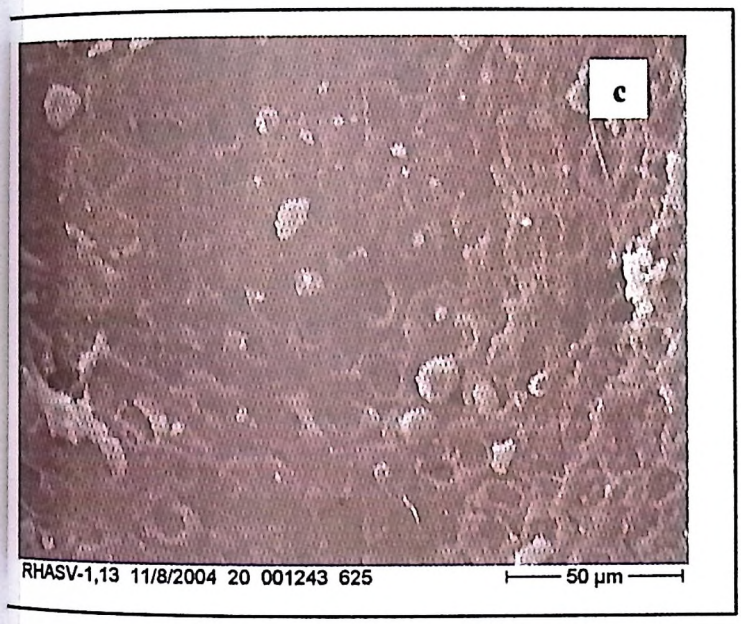
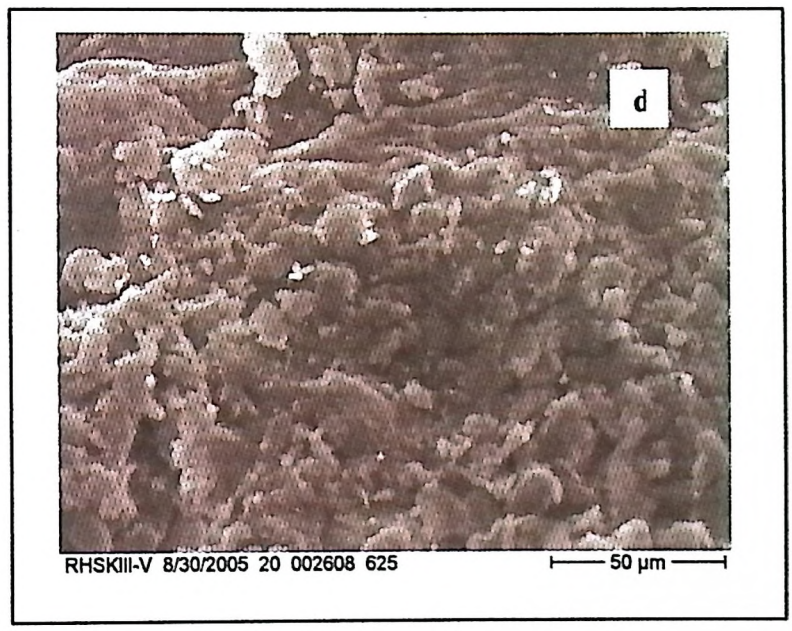


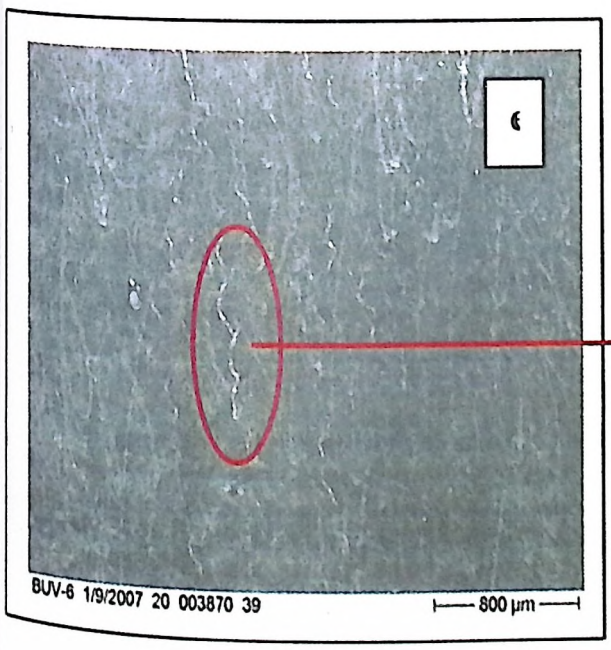
Figure 3.6. Scan micrographs of dorsal portion of horn. a. Rhinoceros; b. Buffalo; c. Fake; d. Fake with hairs.











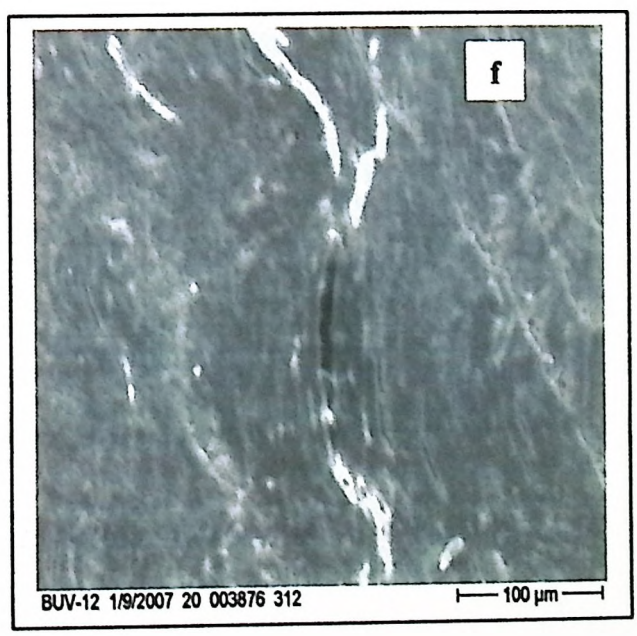
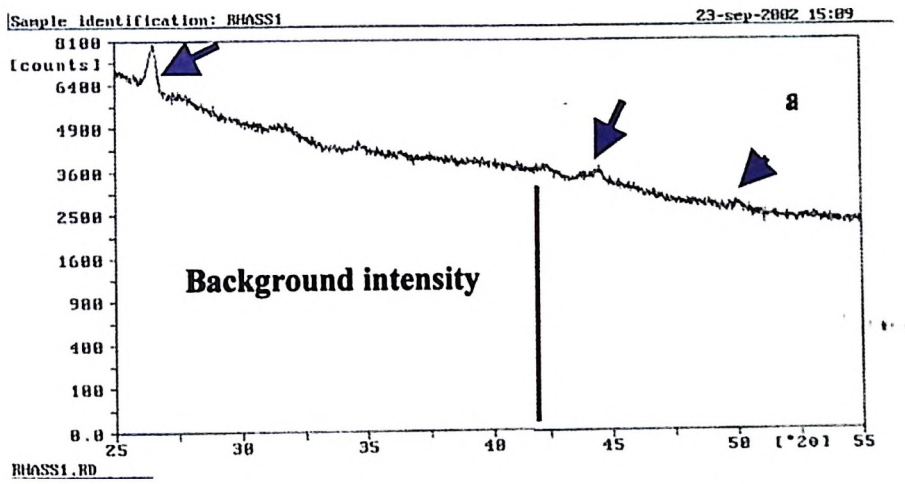


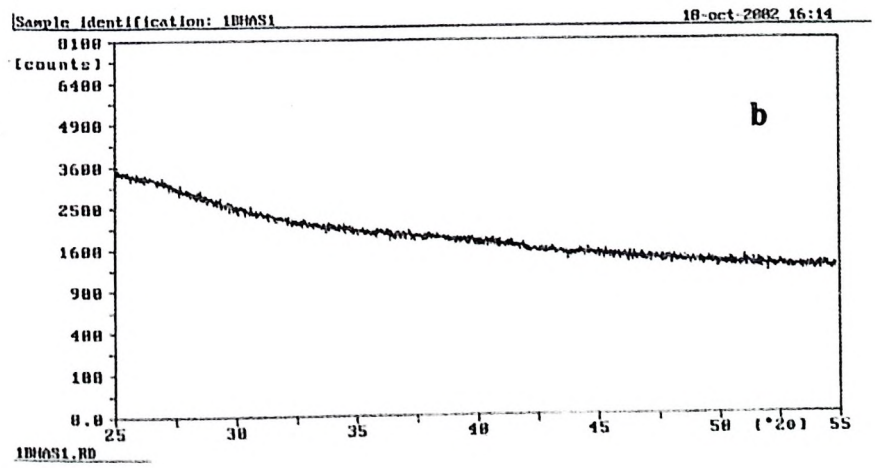
Figure 3.7. Scan micrographs of ventral portion of horn. a. Rhinoceros (n=3); b. Fake (n=3); c. Magnified micrographs of ventral portion of horn; d. Magnified micrographs of fake horn; e. Micrograph of buffalo horn (n=1); f. Magnified micrographs of buffalo horn.

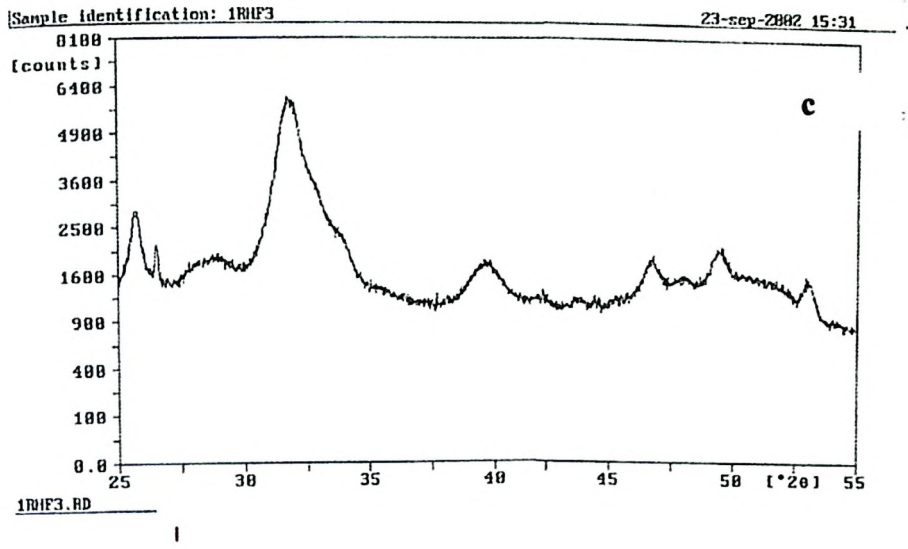
3.5.3. X-ray diffraction characteristics

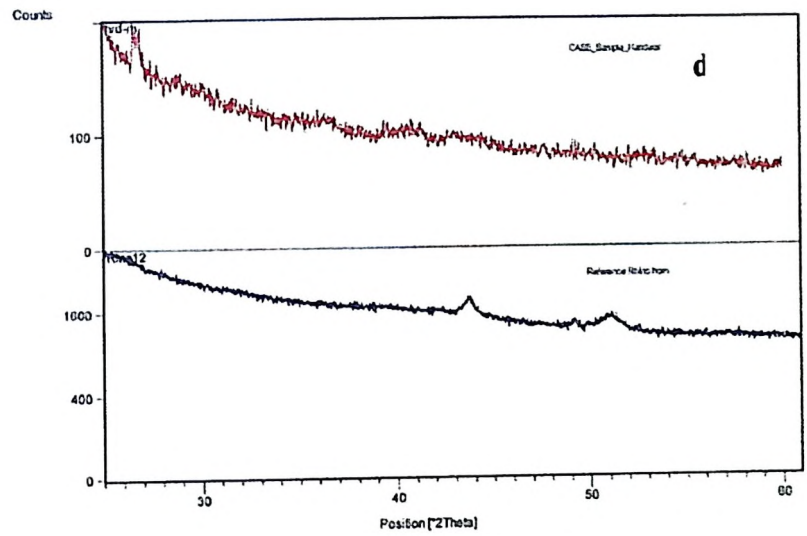
No peak was present in between 5 to 25^0 and after 55 to 100^0 in diffraction pattern of rhinoceros horn. Instead there were three minor characteristic peaks at 26^0, 43^0 and 50^0. Near 50^0 there was a minute shoulder peak at 49^0 (Fig. 3.8a). X-ray diffraction pattern of rhinoceros horn was different from buffalo horn as these minor peaks were not present on the diffractogram of later horn (Fig. 3.8b). Background intensity of rhinoceros horn was higher as compared to buffalo horn (Fig. 3.8a&b).

A fake rhinoceros horn was examined using X-ray diffractometer, the diffractogram was entirely different from rhinoceros horn and matched with bone (Fig. 3.8c). Diffractogram of suspected rhinoceros horn powdered sample was examined and the diffractogram pattern of the powdered sample was entirely different from the rhinoceros horn thus, it gives a clue that the suspected powder was not of rhinoceros horn (Fig. 3.8 d&e).









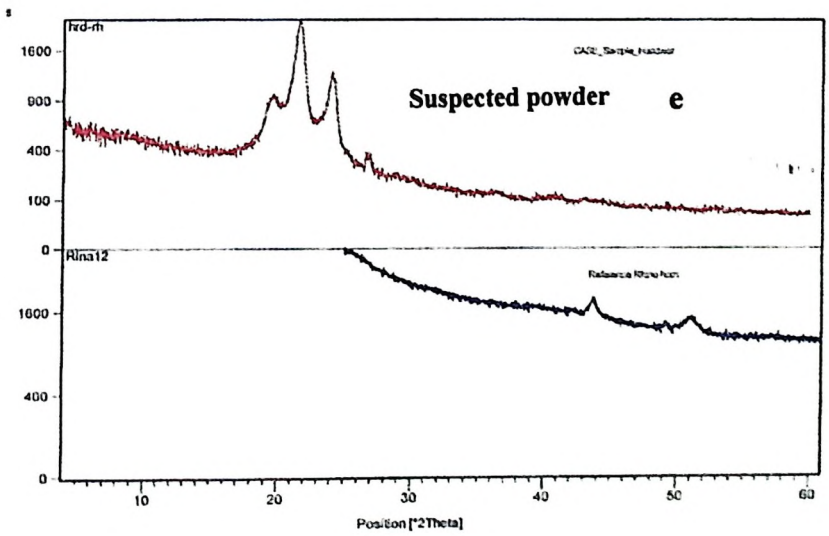
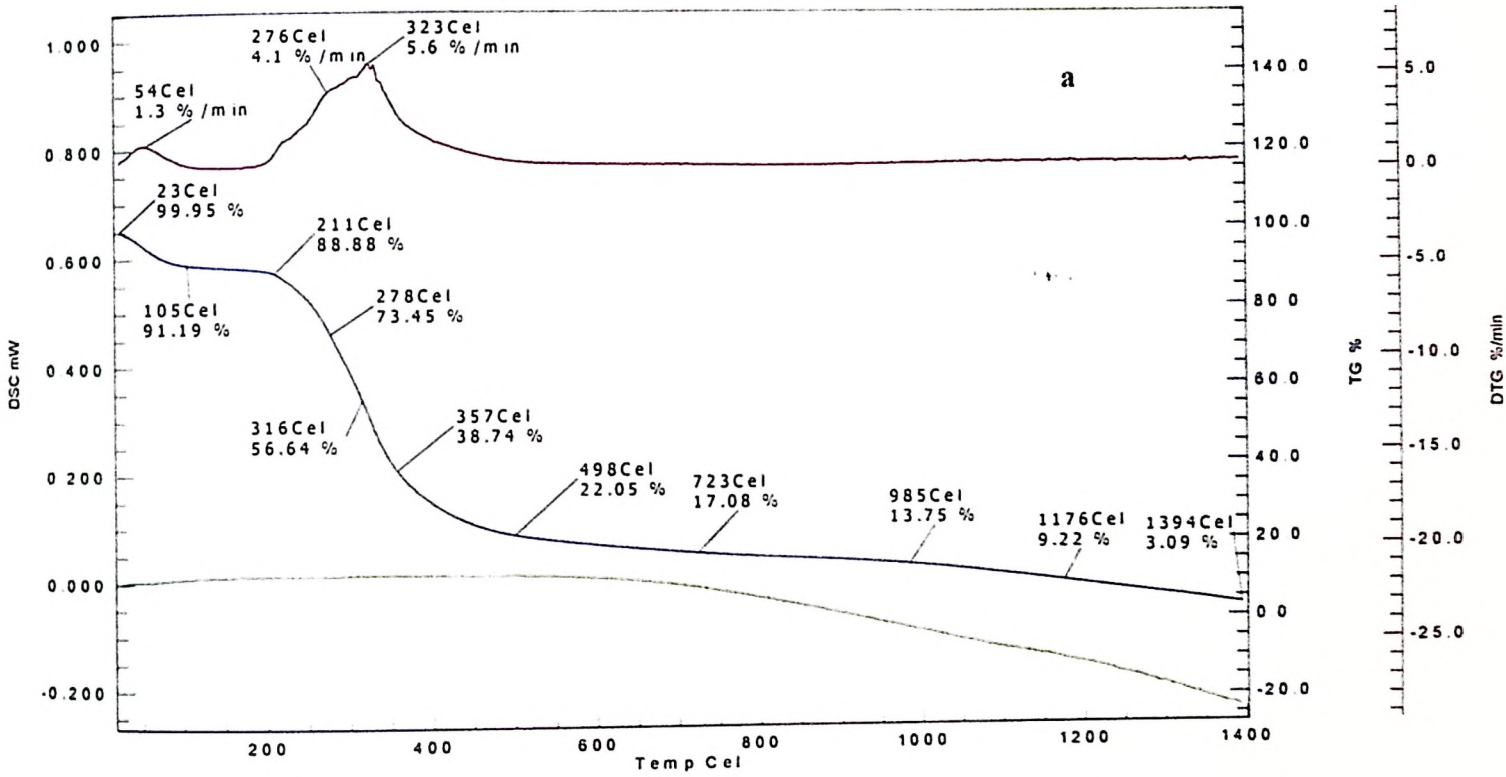
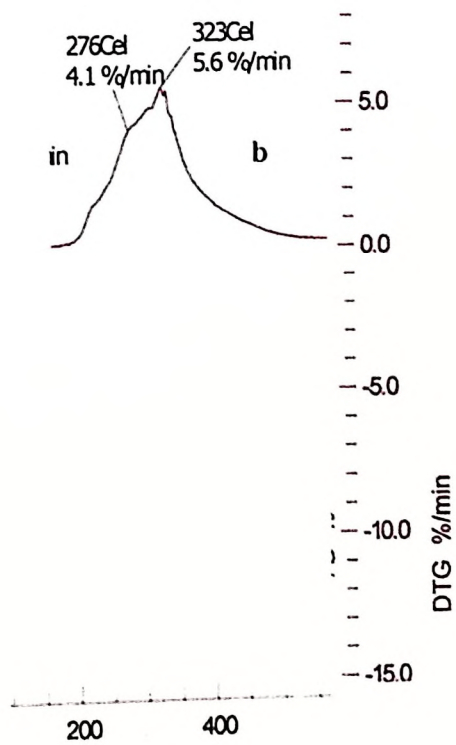


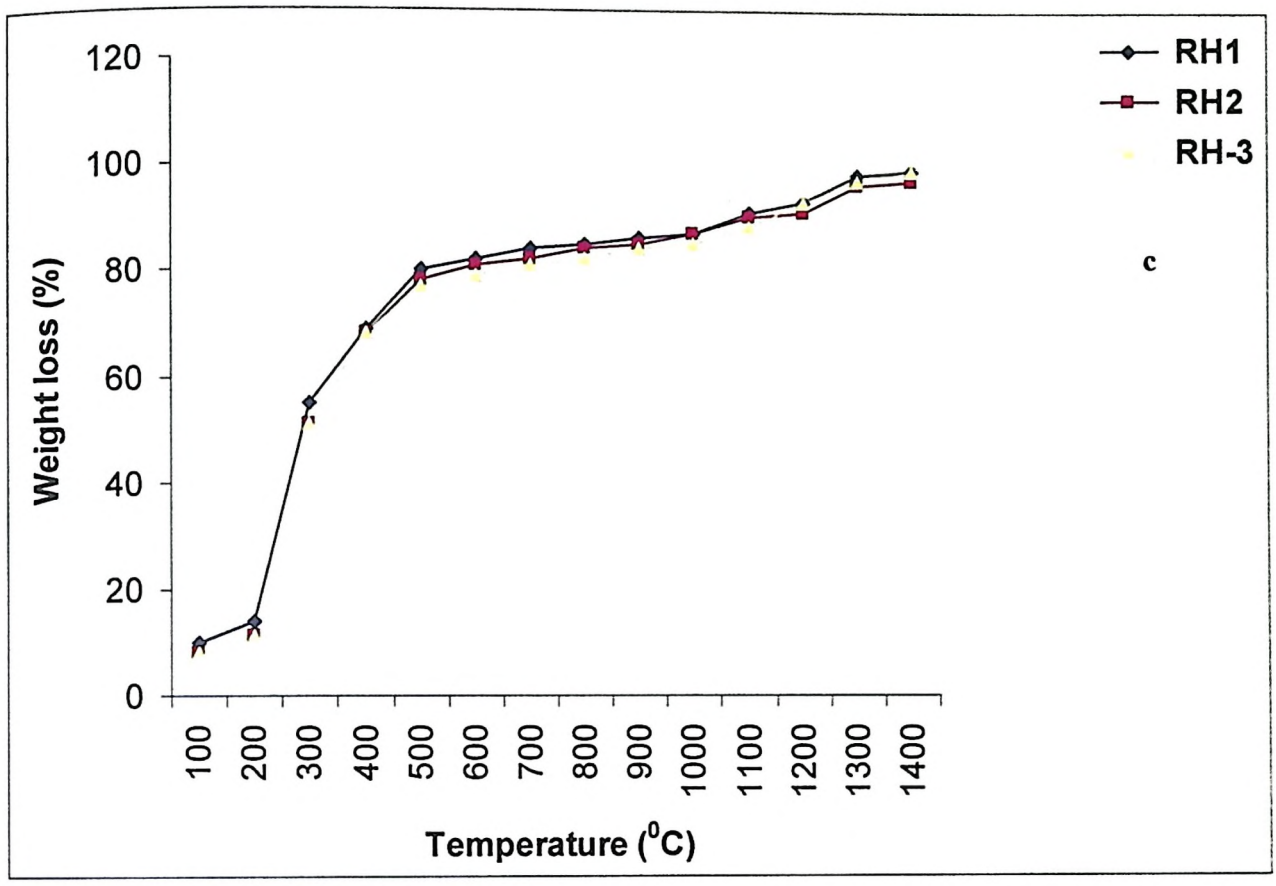
Figure 3.8. Diffractograms of horns. a. Rhinoceros horn; b. Buffalo horn; c. Fake rhinoceros horn; d. Suspected powdered rhinoceros horn; e. Suspected powder showing difference from rhino horn.

3.5.4. Thermo gravimetric analysis (TGA)

Two peaks were present, first between 20 and100^{0}C and second in between 200 and 400^{0}C (Fig.3.9a). A notch is invariably present at the top of the second peak in all thermograph of rhinoceros horns (Fig. 3.9b). Nature of weight loss plotted with constant increase in temperature, shows similar trend in all rhinoceros horns. Maximum weight loss of 65% was between 250 and 550^{0}C (Fig. 3.9). Total weight loss of rhinoceros horns at 1400^{0}C were 97-99% and water loss at 100^{0}C were 8-10% (Fig. 3.9d).







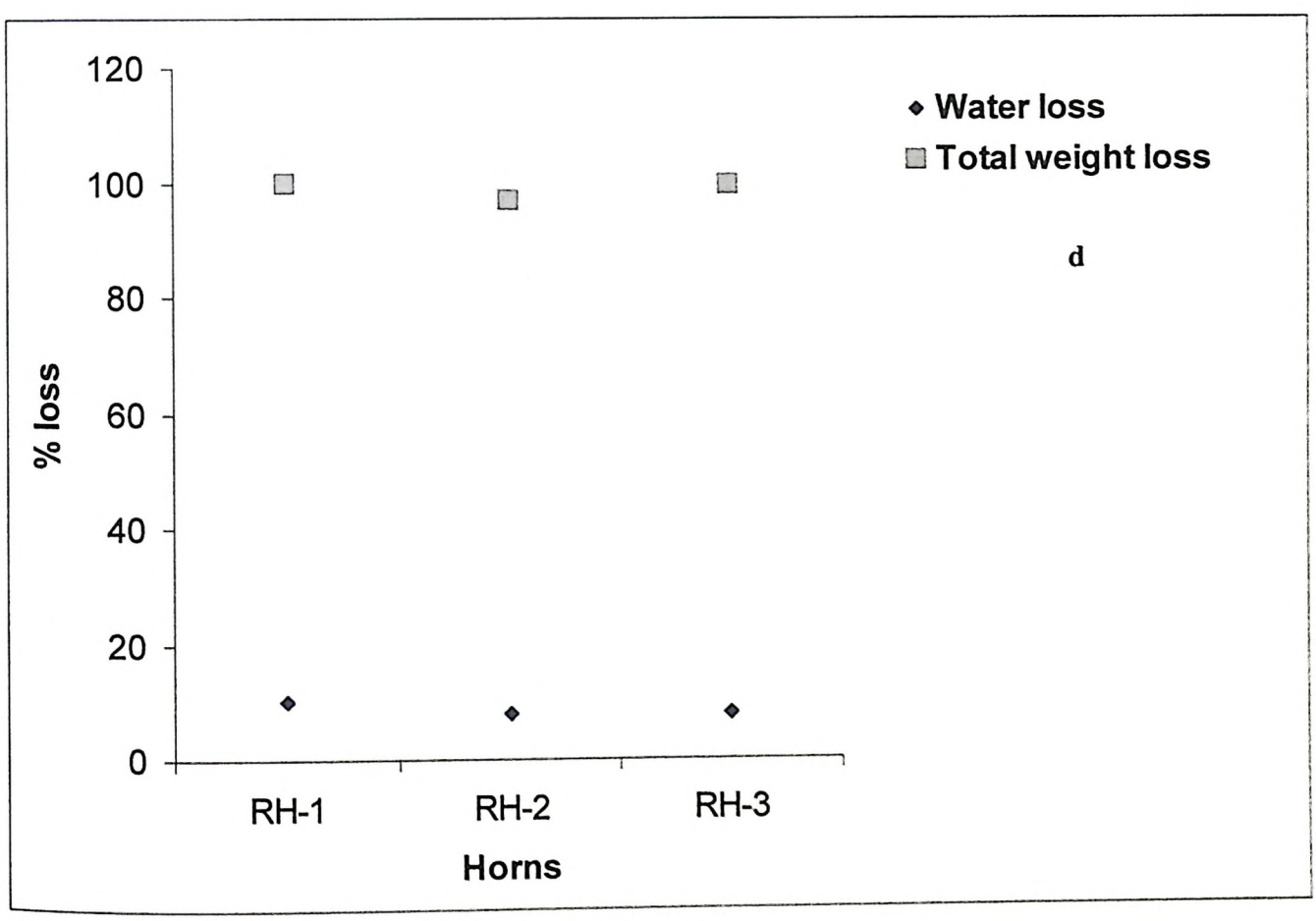


Figure 3.9. a. Thermograph of rhinoceros horn; b. Part of DTG peak of Rhinoceros horn enlarged; c. Trend of percentage weight loss with constant increase in temperature; d. Weight and water loss in Rhinoceros horn.

3.5.5. Elemental analysis

3.5.5.1. X-ray fluorescence

Based on intensities, iron (Fe), phosphorous (P), sulphur (S), zinc (Zn), chlorine (Cl), copper (Cu), chromium (Cr) and nickel (Ni) were high enough to considered as the major/minor constituents where as silicon (Si), aluminum (Al), sodium (Na), magnesium (Mg), and two rare earth elements gadolinium (Gd) and osmium (OS) were trace constituents of the rhinoceros horn based on XRF spectrum (Fig. 3.10). The qualitative elemental fluorescence X-ray scan obtained from rhinoceros horn reveal that the intensity (KCps) of iron (Fe) was highest where as intensity of magnesium (Mg) was lowest among the fourteen elements analyzed.

3.5.5.2. Inductively coupled plasma – mass spectrometry (ICP-MS)

ICP-MS was used to find the quantitative abundance of elements present in rhinoceros horn. Zinc (Zn), iron (Fe), calcium (Ca), potassium (K), magnesium (Mg), uranium (U), lead (Pb), cadmium (Cd), sodium (Na), vanadium (V), nickel (Ni), copper (Cu), cobalt (Co), chromium (Cr), strontium (Sr) and molybdenum (Mo) were analyzed and Fig. 3.11a and Table 3.9 indicate the concentration of these elements in four Asian rhinoceros horns. Three elements viz. Mo, U and Cd were not detectable. The average concentration of various elements in rhinoceros horn, reveals that Ca =67% is most abundant element followed by, Fe=12%, Na=10%, K=4%, Mg=4%, Co=2%, Zn=1% and other elements tested were in traces (Fig. 3.11b). However, mean concentration of four elements Ca, Na, V and Pb were different in wild and the zoo rhinoceros horn. All four elements were of higher concentrations in zoo rhinoceros horn (Fig. 3.11c&d).

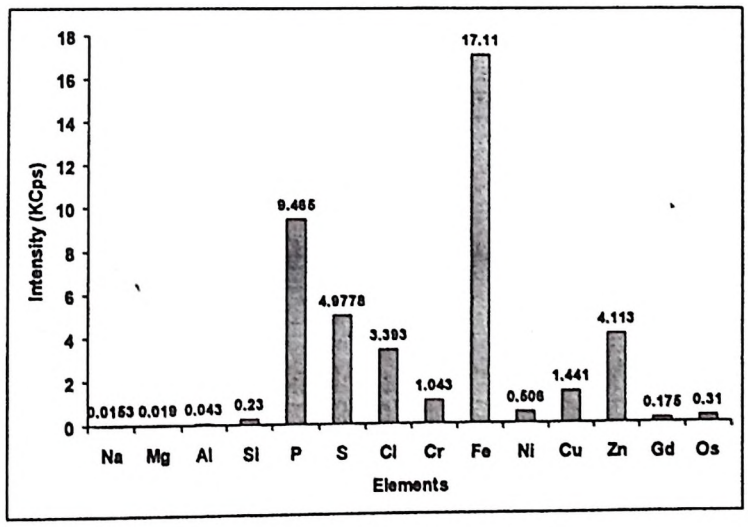
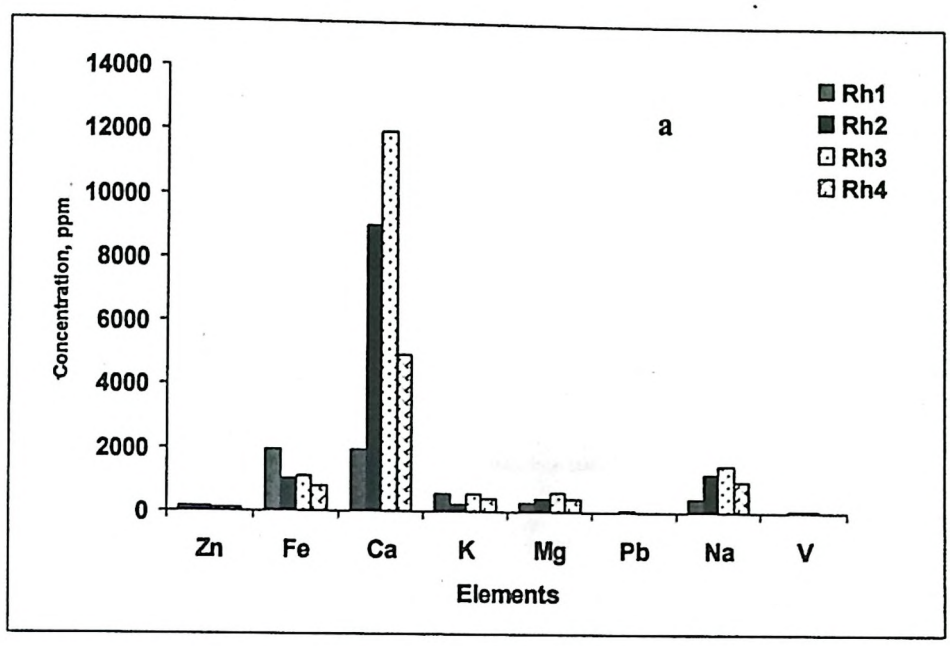
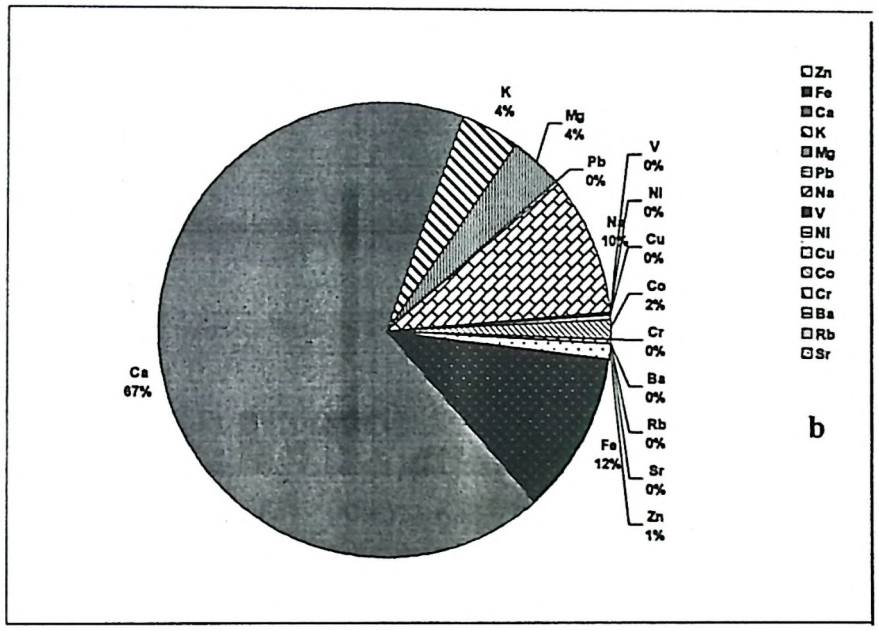


Figure3.10. Intensity of different elements in rhinoceros horn using XRF.


14000
12000
10000
8000
6000
4000
2000
0
Concentration, ppm
Zn
Fe
Ca
K
Mg
Pb
Na
V
Elements
Rh1
Rh2
Rh3
Rh4
a


K 4%
Mg 4%
Pb 0%
V 0%
Ni 0%
Na 10%
Cu 0%
Co 2%
Cr 0%
Ba 0%
Rb 0%
Sr 0%
Zn 1%
Fe 12%
Ca 67%
Zn
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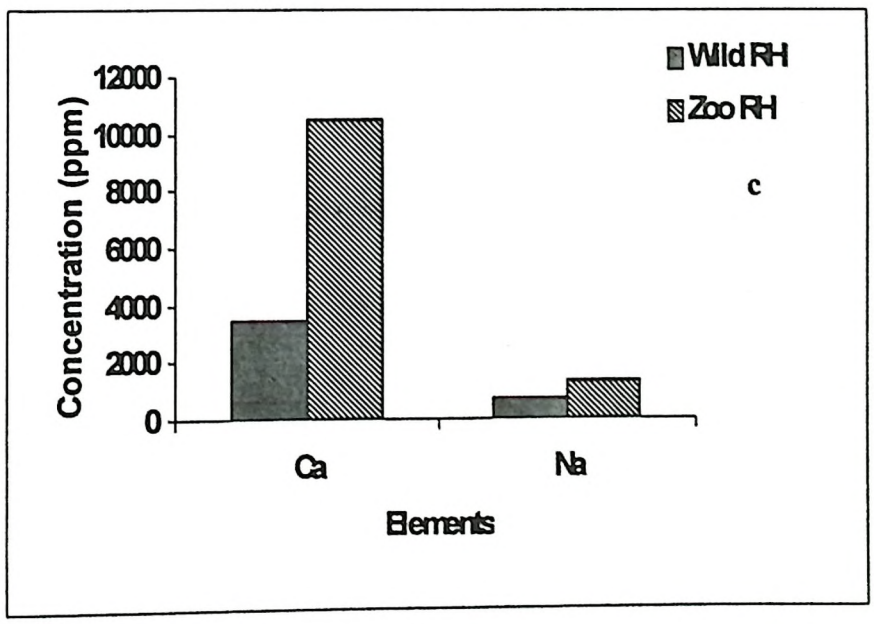
Na
V
Ni
Cu
Co
Cr
Ba
Rb
Sr
b


12000
10000
8000
6000
4000
2000
0
Concentration (ppm)
Ca
Na
Elements
Wild RH
Zoo RH
c

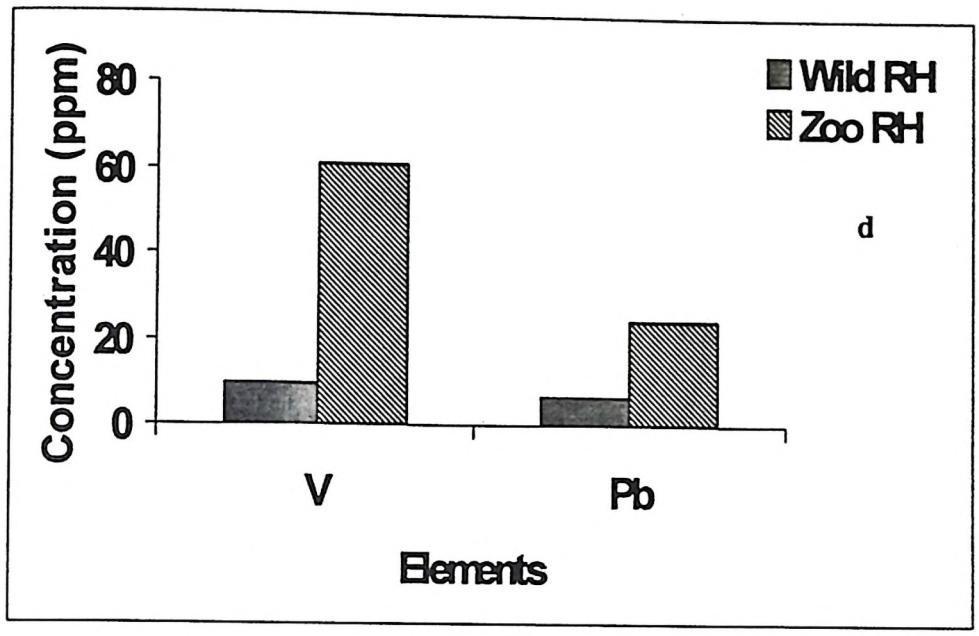


Figure3.11. Variation in the element concentration determined based on ICP-MS in great one horned rhinoceros horn. a. Concentration of Zn, Fe, Ca, K, Mg, Pb, Na and V; b. Percentage occurrence of various elements; c. Concentration of calcium and sodium in rhinoceros horn from wild and zoo; d. Concentration of vanadium and lead in rhinoceros horn from wild and zoo.

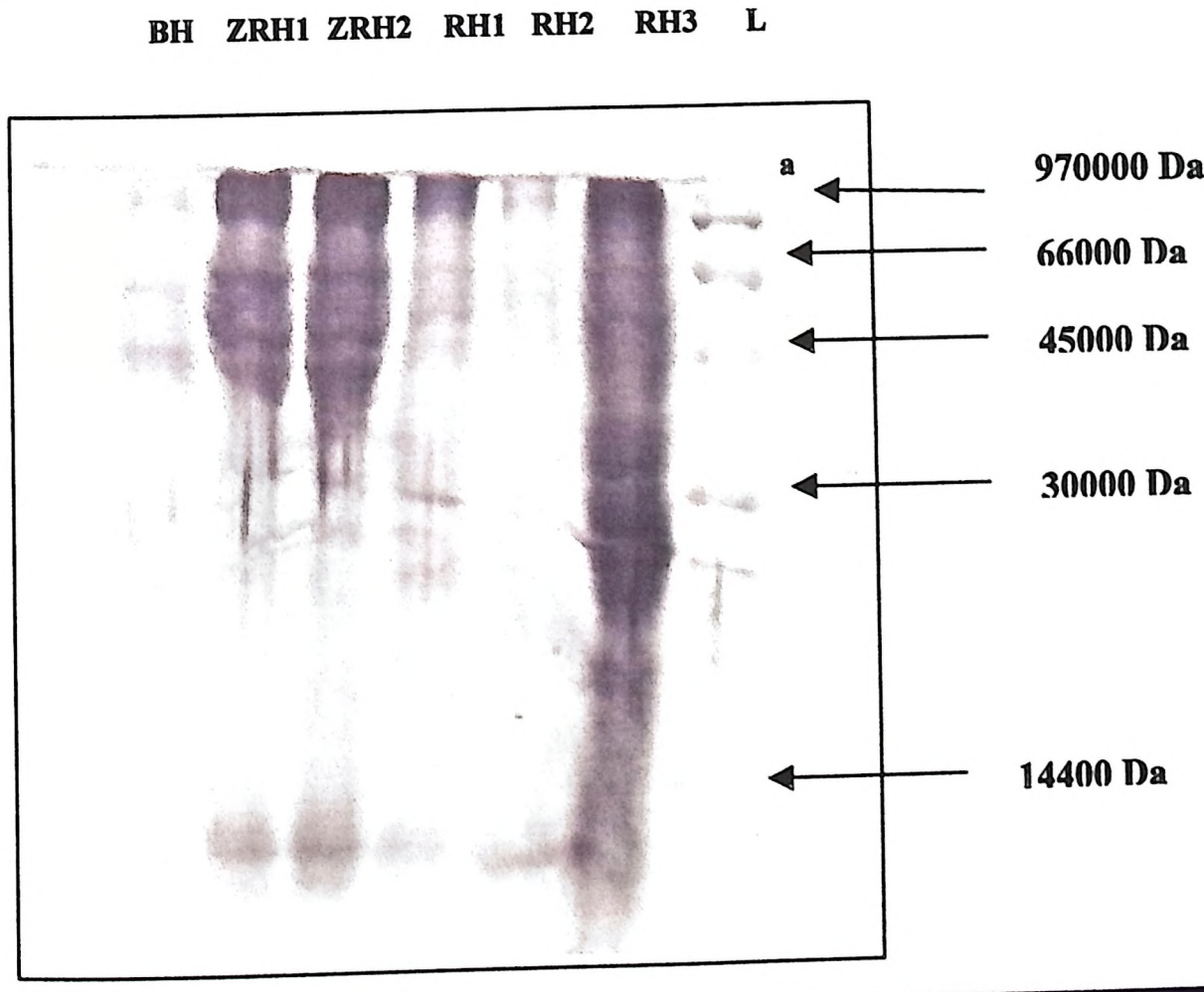
Table 3.9. Concentrations of elements in ppm of rhinoceros horns analyzed through ICP-MS.

Elements	Mean (ppm)	St. Deviation
Zn	117.38	23.69
Fe	1217.94	507.99
Ca	6969.34	4427.20
K	437.0	148.24
Mg	412.67	126.05
U	0	0
Pb	15.37	10.59
Cd	0	0
Na	1007.24	448.99
V	35.27	29.88
Ni	5.46	5.17
Cu	10.17	7.88
Co	120.56	101.89
Cr	6.45	6.56
Ba	2.48	4.95
Rb	1.24	0.95
Sr	6.70	4.54
Mo	0	0

3.5.6. Protein profile

Sodium Dodicyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE) was run to examine the number of proteins bands (PB) present at different positions according to their molecular weights and was calibrated using low molecular weight SDS kit, in rhinoceros horn (PB=5-8), buffalo horn (PB=6) and fake rhinoceros horn (PB=0) (Fig. 3.12a). The band positions in horns of rhinoceros and buffalo were different. There was a band between 66000 – 45000 Dalton in rhinoceros horn which was absent in buffalo horn. There were two bands between 45000-30000 Dalton in rhinoceros horn but, in buffalo horn there was only a band which had higher molecular weight. There were 1 - 2 bands below 14400 in rhinoceros horn where as there were three closely placed bands after 14400 in buffalo horn. In zoo rhinoceros horn, the first band was much higher than the first band of rhinoceros horn from wild and buffalo horn. In rhinoceros horns from zoo there was a band at about 28000-27000, which was absent in the rhinoceros horns from wild. In wild the band was either above 30000 or below 20000 (Table 3.11&3.12).

Based on the molecular weight calculated a dendrogram was generated using Ward method to cluster rhinoceros horn and buffalo horn which clearly differentiate rhinoceros horns from buffalo horn based on presence of protein bands at different positions (Fig. 3.12b).



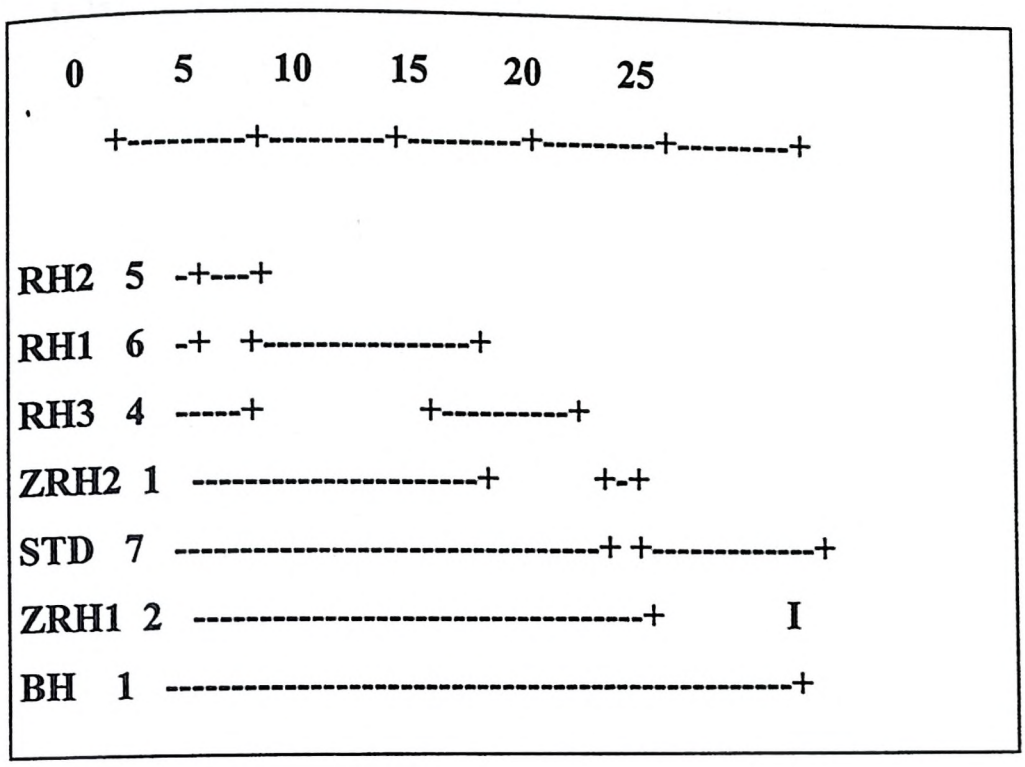


Figure 3.12. Protein profile and cluster analysis of the band observed. a. Resolved protein bands; b. Output of cluster analysis based on Ward's method. RH= Rhino horn; ZRH= Zoo rhino horn; BH= Buffalo.

Table 3.10. Molecular weight (Dalton) of proteins found in horns.

Band	BH	ZRH-1	ZRH-2	RH-3	RH-2	RH-1
1	108688	117594	110607	104949	97853	100456
2	43466	46628	46444	47935	47183	45897
3	29779	38963	33378	37136	38344	35871
4	5118	32936	27320	30811	30976	30241
5	3599	28278	2941	10327	13708	19883
6	2704	3059		2948	2827	16776
7						11010
						2738

RH, Rhino horn; ZRH, Zoo rhino horn; BH, Buffalo horn

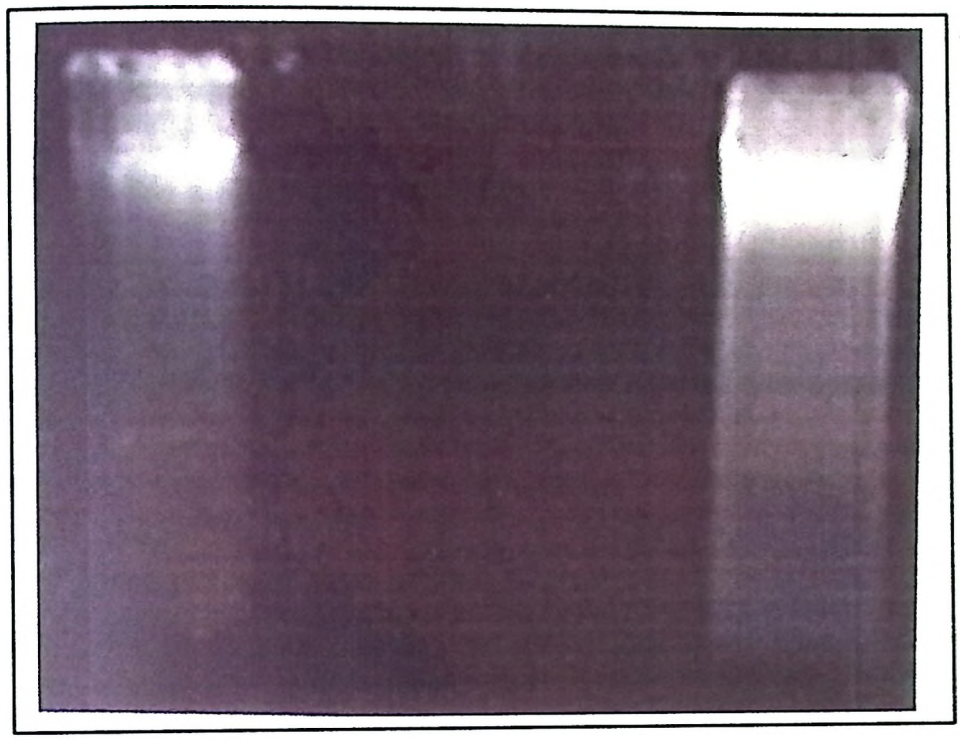
Table 3.11. Number of proteins bands found in rhinoceros and buffalo horns.

Mol. Wt. (Dalton) protein	Number of protein bands		
	Rhinoceros horn		Buffalo horn (n=1)
	Wild (n=3)	Zoo (n=2)	
97000-66000	1	1	1
66000-45000	1	1	0
45000-30000	2	1-2	1
30000-14400	0-2	1	1
<14400	1-2	1	3

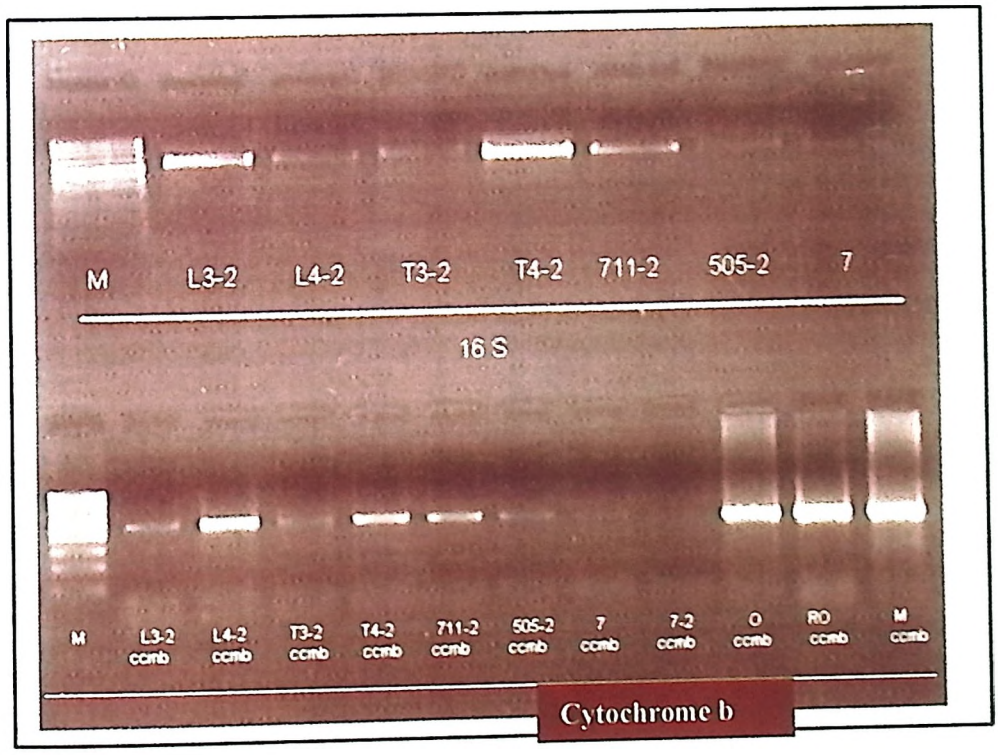
3.5.7. DNA techniques

Scraping of the sample gives better result and any portion of basal region could be used for extracting DNA, however tip was not tried for extraction. Minimum 25 mg of sample is required to extract DNA from rhinoceros horn and sample amount can be increased to get better yield. Overnight digestion is sufficient but time can be increased to 36 hours which will help in better digestion of samples and will yield higher amount of DNA. 0.39 mM DTT was used in digestion and it gave better result. DNA was extracted from rhinoceros horns using Qiagen protocol and it yielded 150bp to 600bp of DNA (Fig. 3.13a). Cleaning of DNA is better to remove contamination but, it depends on the amount of DNA eluted, if there is less amount of DNA it will result in further loss. Thus, purification of DNA can be tried when ample amount of DNA is present.

PCR reactions tried with cytochrome b 450 bp and 16S rRNA 550 bp were successfully amplified (Fig. 3.13b). In Fig. 3.11b the lanes marked RH and RH-2 were for amplification of rhinoceros horn sample using both primers. Further sequencing of these PCR products may be done for identification purpose.



a. Quality of DNA



b. PCR amplification with cytochrome b and 16S rRNA primers

Figure 3. 13. Quality of DNA and PCR amplification of rhinoceros horn (7).

```
                                      10        20        30        40        50
                                      ....|....|....|....|....|....|....|....|....|
NC_001779 Rhinoceros unicornis    1   CATCAACCACTCATTCATCGACCTACCTACCCCATCAAACATCTCATCTT
DQ470777 Bos taurus               1   TG.A...A.TG.............T..AG.............T.....A.
DQ470776 Bos taurus               1   TG.A...A.TG.............T..AG.............T.....A.
DQ480078 Bubalus bubalis          1   TC.A...A.TG.......T.....C...G.T..................A.
DQ480077 Bubalus bubalis          1   TC.A...A.TG.......T.....C...G.T..................A.
DQ480076 Bubalus bubalis          1   TC.A...A.TG.......T.....C...G.T..................A.
DQ480075 Bubalus bubalis          1   TC.A...A.TG.......T.....C...G.T..................A.

                                      60        70        80        90        100
                                      ....|....|....|....|....|....|....|....|....|....|
NC_001779 Rhinoceros unicornis    51  GATGAAACTTTGGCTCCCTGTTAGGAATCTGCTTAATCTTACAGATCCTA
DQ470777 Bos taurus               51  .......T..C..T.....CC.G.........C.....C....A.....C
DQ470776 Bos taurus               51  .......T..C..T.....CC.G.........C.....C....A.....C
DQ480078 Bubalus bubalis          51  ................T..CC....C......C....TC.G..A.....C
DQ480077 Bubalus bubalis          51  ................T..CC....C......C....TC.G..A.....C
DQ480076 Bubalus bubalis          51  ................T..CC....C......C.....C.G..A.....C
DQ480075 Bubalus bubalis          51  ................T..CC....C......C....TC.G..A.....C

                                      110       120       130       140       150
                                      ....|....|....|....|....|....|....|....|....|....|
NC_001779 Rhinoceros unicornis    101 ACAGGACTATTCCTTGCCATACACTACACCCCAGACACAACAACCGCCTT
DQ470777 Bos taurus               101 .....C........A..A...........AT.C...........A..A..
DQ470776 Bos taurus               101 .....C........A..A...........AT.C...........A..A..
DQ480078 Bubalus bubalis          101 ..C..C........A..A...........AT.C...........A..A..
DQ480077 Bubalus bubalis          101 ..C..C........A..A...........AT.C...........A..A..
DQ480076 Bubalus bubalis          101 ..C..C........A..A...........AT.C...........A..A..
DQ480075 Bubalus bubalis          101 ..C..C........A..A...........AT.C...........A..A..

                                      160       170       180       190       200
                                      ....|....|....|....|....|....|....|....|....|....|
NC_001779 Rhinoceros unicornis    151 TTCATCCGTCACCCATATCTGCCGAGACGTAAATTACGGCTGAATAATTC
DQ470777 Bos taurus               151 C..C..T..T.....................G..C...........C..C.
DQ470776 Bos taurus               151 C..C..T..T.....................G..C...........C..C.
DQ480078 Bubalus bubalis          151 C..C..T...G....C........G.....G..C..T..A.....T....
DQ480077 Bubalus bubalis          151 C..C......G....C........G.....G..C..T..A.....T....
DQ480076 Bubalus bubalis          151 C..C......G....C........G.....G..C..T..A.....T....
DQ480075 Bubalus bubalis          151 C..C..T...G....C........G.....G..C..T..A.....T....

                                      210       220       230       240       250
                                      ....|....|....|....|....|....|....|....|....|....|
NC_001779 Rhinoceros unicornis    201 GCTACCTCCATGCCAACGGAGCATCCATATTCTTCATCTGCCTATTTATT
DQ470777 Bos taurus               201 .A...A.A..C..A........T..A..G..T..T......T...A...G
DQ470776 Bos taurus               201 .A...A.A..C..A........T..A..G..T..T......T...A...G
DQ480078 Bubalus bubalis          201 .A...A.A..C..A........T..A.....T.........T...A...A
DQ480077 Bubalus bubalis          201 .A...A.A..C..A........T..A.....T.........T...A...A
DQ480076 Bubalus bubalis          201 .A...A.A..C..A........T..A.....T.........T...A...A
DQ480075 Bubalus bubalis          201 .A...A.A..C..A........T..A.....T.........T...A...A

                                      260       270       280       290       300
                                      ....|....|....|....|....|....|....|....|....|....|
NC_001779 Rhinoceros unicornis    251 CATGTAGGACGAGGCCTTTACTATGGATCTTACACCTTCCTAGAAACTTG
DQ470777 Bos taurus               251 ..C............T.A..T..C..G........T..T........A..
DQ470776 Bos taurus               251 ..C............T.A..T..C..G........T..T........A..
DQ480078 Bubalus bubalis          251 ..C............A.A.....C.....A..T.....T........A..
DQ480077 Bubalus bubalis          251 ..C............A.A.....C.....A..T.....T........A..
DQ480076 Bubalus bubalis          251 ..C............A.A.....C.....A..T.....T........A..
DQ480075 Bubalus bubalis          251 ..C............A.A.....C.....A..T.....T........A..
```

```
                                    310        320        330        340        350
                                    ....|....|....|....|....|....|....|....|....|....|
NC_001779 Rhinoceros unicornis      301 AAACATTGGAATTATCCTACTATTTACCCTAATAGCCACAGCGTTCATAG
DQ470777 Bos taurus                 301 ...T......G.A.....T..GC.C..AG.............A..T....
DQ470776 Bos taurus                 301 ...T......G.A.....T..GC.C..AG.............A..T....
DQ480078 Bubalus bubalis            301 ......C...G.A...........CG.AG.............A..T....
DQ480077 Bubalus bubalis            301 ......C...G.A...........CG.AG.............A..T....
DQ480076 Bubalus bubalis            301 ......C...G.A...........CG.AG.............A..T....
DQ480075 Bubalus bubalis            301 ......C...G.A...........CG.AG...........G..A..T....

                                    350
                                    ....|....|....|
NC_001779 Rhinoceros unicornis      351 GTTACGTCCTACCAT
DQ470777 Bos taurus                 351 .A.............
DQ470776 Bos taurus                 351 .A.............
DQ480078 Bubalus bubalis            351 .A.....A..G....
DQ480077 Bubalus bubalis            351 .A.....A..G....
DQ480076 Bubalus bubalis            351 .A.....A..G....
DQ480075 Bubalus bubalis            351 .A.....A..G....
```

Figure 3.14. DNA sequence alignment of *Rhinoceros unicornis* at cytochrome b region with *Bos taurus* and *Bubalus bubalis.*

In present study partial sequences of a mitochondrial cytochrome b genes was analysed. Alignment of one DNA sequence of *Rhinoceros unicornis* with, two sequences of *Bos taurus* and 4 sequences of *Bubalus bubalis* indicates the most variable region conserved within species, which are highly variable between species (Fig. 3.14). This will help to develop specific specific primers. Sequences of cytochrome b *Rhinoceros unicornis*, *Bubalus bubalis* and Bos *taurus* were aligned using Bioedit software. Analysis of sequences revealed that more then 24.65 % (90 sites) sites were polymorphic out of 365bp of cytochrome b alignment.

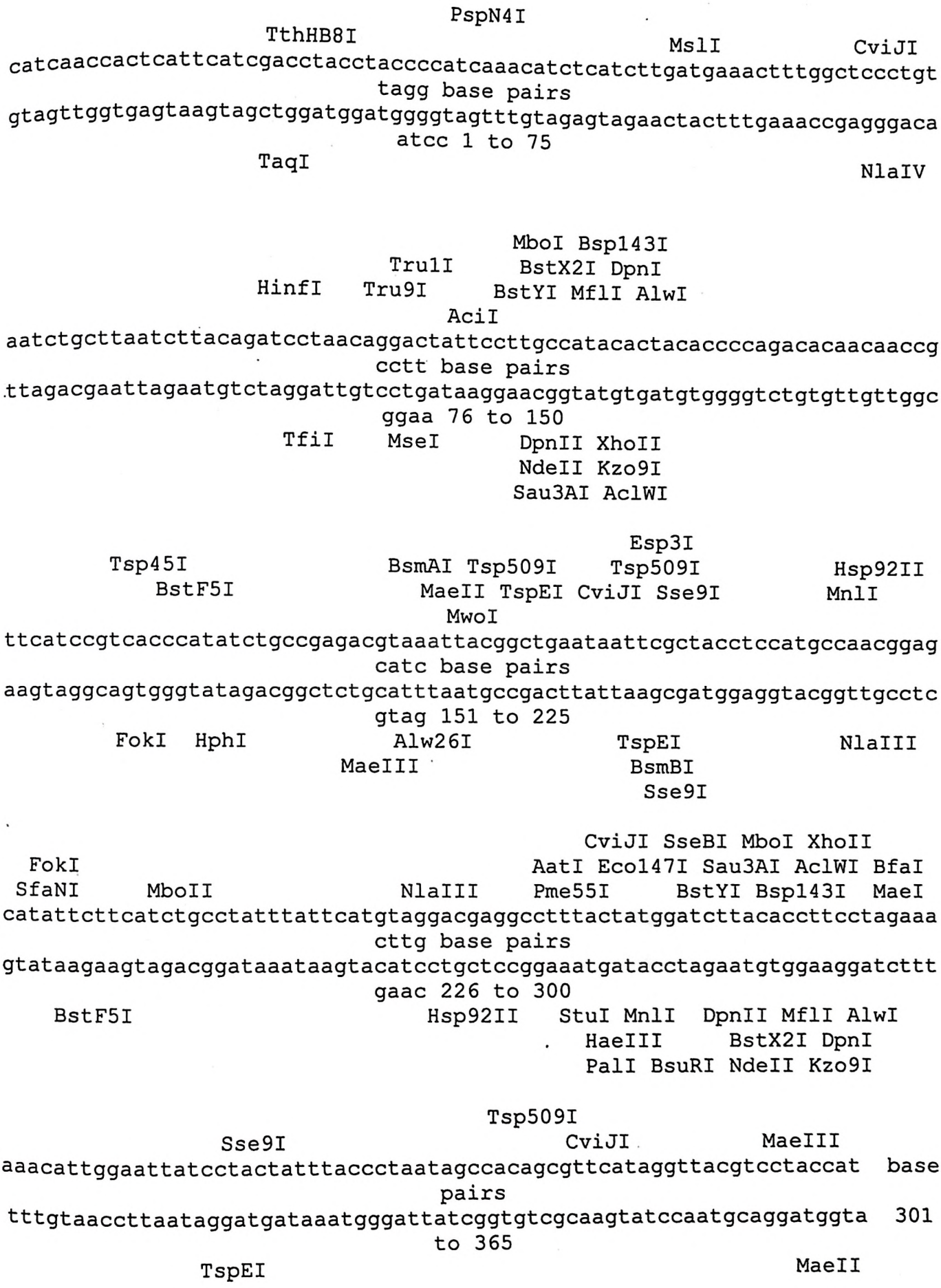


Figure 3.15.Restriction map of *Rhinoceros unicornis* for cytochrome b gene.

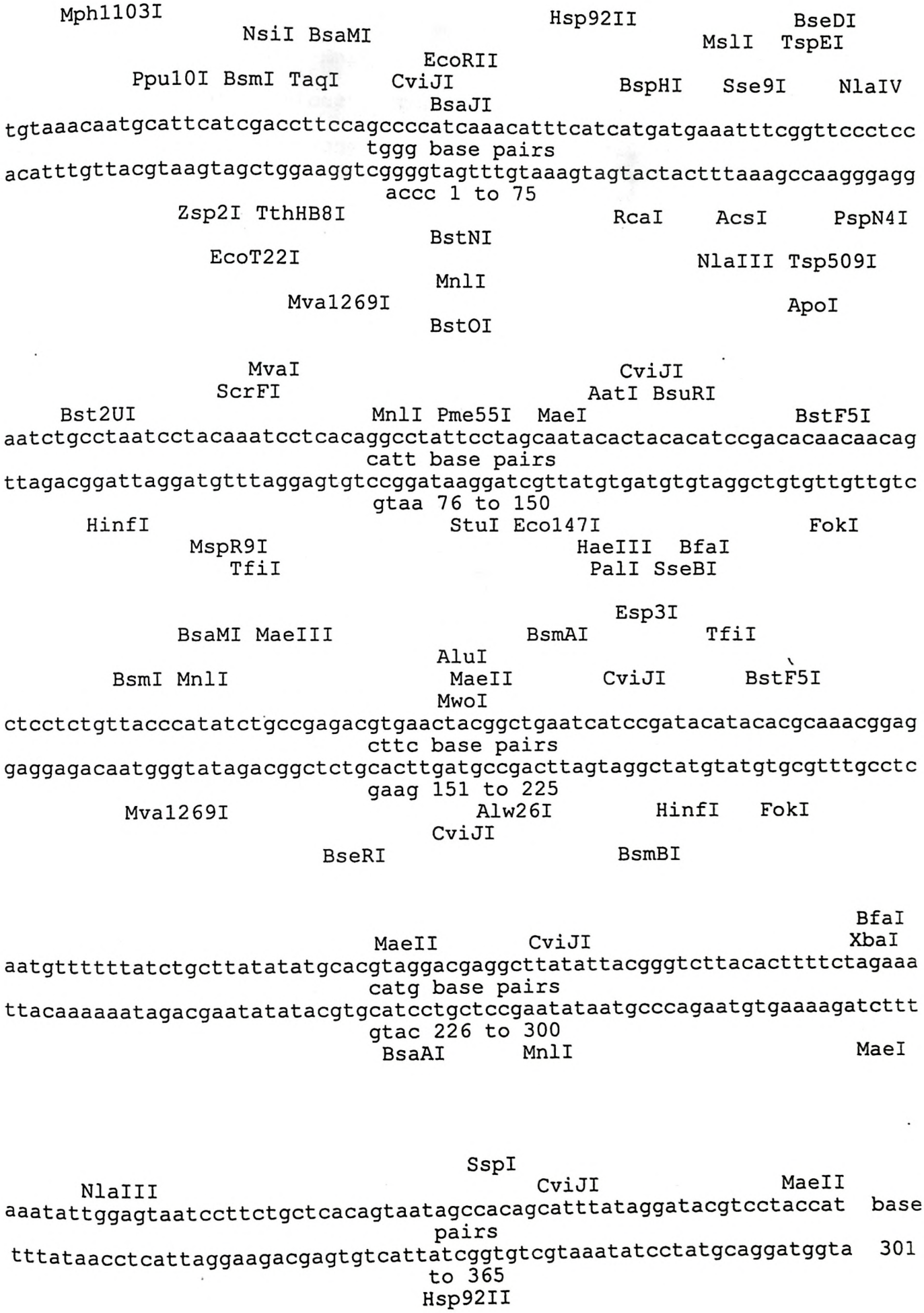


Figure 3.16. Restriction map of *Bos taurus* for cytochrome b gene.

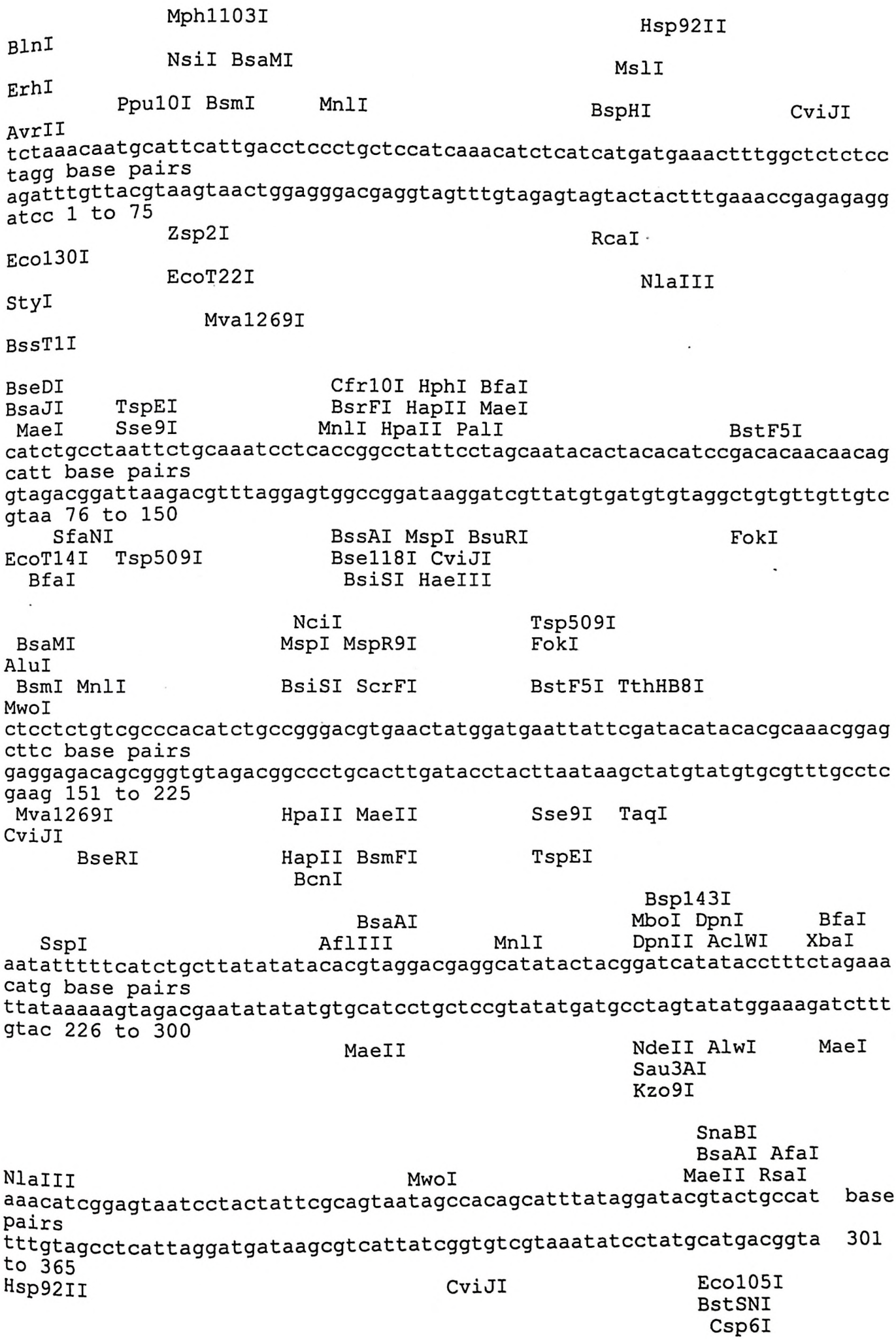


Figure 3.17. Restriction map of *Bubalus bubalis* for cytochrome b gene.

Table 3.12. Restriction enzymes cutting partial fragments of cytochrome b gene of rhinoceros and buffalo.

Enzyme	Recogniti on sequence	*Rhinoceros unicornis*		*Bos taurus*		*Bubalus bubalis*	
		No. of cuts	Positions of sites	No. of cuts	Positions of sites	No. of cuts	Positions of sites
AatI	agg/cct	1	264	1	105	-	-
AclWI	ggatc	-	-	-	-	1	279
AcsI	r/aatty	-	-	1	55	-	-
AfaI	gt/ac	-	-	-	-	1	357
AvrII	c/ctagg	-	-	-	-	1	70
AflIII	a/crygt	-	-	-	-	1	250
ApoI	r/aatty	-	-	1	55	-	-
Alw26I	gtctc	-	-	1	178	-	-
BfaI	c/tag	-	-	2	113 290	3	71 113 290
BstX2I	**r/gatcy**	**2**	**93 275**	-	-	-	-
BstYI	**r/gatcy**	**2**	**93 275**	-	-	-	-
BsaAI	yac/gtr	-	-	1	253	2	253 355
BsaJI	c/cnngg	-	-	1	70	2	70 336
BseDI	c/cnngg	-	-	1	70	2	70 336
BsmAI	gtctc	-	-	1	178	-	-
Bst2UI	cc/wgg	-	-	1	71	-	-
BcnI	cc/sgg	-	-	-	-	1	173
Bse118I	r/ccggy	-	-	-	-	1	101
BsiSI	c/cgg	-	-	-	-	2	102 172
BsmFI	gggac	-	-	-	-	1	178
Bsp143I	/gatc	-	-	-	-	1	275
BsrFI	r/ccggy	-	-	-	-	1	101
BssAI	r/ccggy	-	-	-	-	1	101
BssT1I	c/cwwgg	-	-	-	-	1	70
BstDSI	c/crygg	-	-	-	-	1	336
BstF5I	ggatg	-	-	2	133 200	2	133 192
BstNI	cc/wgg	-	-	1	71	1	355
BstOI	cc/wgg	-	-	1	71	-	-
BlnI	c/ctagg	-	-	-	-	1	70
CviJI	rg/cy	-	-	6	29 105 189 221 264 335	4	63 105 221 335
Cfr10I	r/ccggy	-	-	-	-	1	101
Csp6I	g/tac	-	-	-	-	1	356
DpnI	ga/tc	-	-	-	-	1	277
DpnII	/gatc	-	-	-	-	1	275
DsaI	c/crygg	-	-	-	-	1	336
Eco147I	agg/cct	1	264	1	105	-	-
Eco105I	tac/gta	-	-	-	-	1	355
EcoRII	/ccwgg	-	-	1	69	-	-

Eco130I	c/cwwgg	-	-	-	-	1	70
EcoT14I	c/cwwgg	-	-	-	-	1	70
ErhI	c/cwwgg	-	-	-	-	1	70
FokI	ggatg	-	-	2	133 200	2	133 192
HapII	c/cgg	-	-	-	-	2	102 172
HpaII	c/cgg	-	-	-	-	2	102 172
HphI	ggtga	-	-	-	-	1	103
HinfI	g/antc	-	-	-	-	2	75 192
MflI	**r/gatcy**	**2**	**93 275**	-	-	-	-
Kzo9I	/gatc	-	-	-	-	1	275
MaeI	c/tag	-	-	2	113 290	3	71 113 290
MboI	/gatc	-	-	-	-	1	275
MaeIII	/gtnac	-	-	1	157	-	-
MnlI	cctc	-	-	4	70 100 156 264	4	25 100 156 264
MspR9I	cc/ngg	-	-	1	71	-	-
MspI	c/cgg	-	-	-	-	2	102 172
MspR9I	cc/ngg	-	-	-	-	1	173
MvaI	cc/wgg	-	-	1	71	-	-
MwoI	gcnnnnn/nngc	-	-	1	218	2	218 332
Pme55I	agg/cct	1	264	1	105	-	-
SseBI	**agg/cct**	**1**	**264**	-	-	-	-
StuI	agg/cct	1	264	1	105	-	-
XhoII	**r/gatcy**	**2**	**93 275**	-	-	-	-
NlaIV	ggn/ncc	-	-	1	64	-	-
NciI	cc/sgg	-	-	-	-	1	173
NdeII	/gatc	-	-	-	-	1	275
PspN4I	ggn/ncc	-	-	1	64	-	-
RsaI	gt/ac	-	-	-	-	1	357
ScrFI	cc/ngg	-	-	1	71	1	173
Sau3AI	/gatc	-	-	-	-	1	275
SfaNI	gcatc	-	-	-	-	1	79
SnaBI	tac/gta	-	-	-	-	1	355
Sse9I	/aatt	-	-	-	-	2	84 192
SseBI	agg/cct	-	-	1	105	-	-
SspI	aat/att	-	-	1	304	1	228
StyI	c/cwwgg	-	-	-	-	1	70
TaqI	t/cga	-	-	1	18	1	199
Tsp509I	/aatt	-	-	-	-	2	84 192
TfiI	g/awtc	-	-	2	75 192	-	-
Tsp509I	/aatt	-	-	1	55	-	-
TspEI	/aatt	-	-	1	55	2	84 192
TthHB8I	t/cga	-	-	1	18	1	199

Restriction enzymes which cut the cytochrome b gene fragment at different sites in rhinoceros and buffalo are listed in Fig. 3.15 to 3.17 and Table 3.12. These can be used to differentiate species from one another. Five restriction enzymes cuts only rhinoceros DNA fragments at various positions are indicated in bold letters (Table 3.12). Fifteen restriction enzymes cuts *Bos taurus* DNA sequences at various positions. Forty-nine restriction enzymes specific to *Bubalus bubalis* DNA sequences. No restriction enzyme were common to all three species.

Primers were designed of 210-250 bp to be used for all these three species (Table 3.13).

Table 3.13. Primers developed for *Rhinoceros unicornis*, *Bos taurus* and *Bubalus bubalis* DNA using Primer 3 software of cytochrome b region.

Primer		Fragment size	Tm	GC contents
Forward	Reverse			
CTTTGGCTCCCT GTTAGGAA	AAAGGCCTCGTCCTAC ATGA	212	59.5	50
TTGGCTCCCTGTTAGGA ATC	AAAGGCCTCGT CCTACATGA	210	59.4	50
CGACCTACCTACCCCAT CAA	AAGGCCTCGTC CTACATGAAT	250	59.9	51.3
TGAAACTTTGGC TCCCTGTT	AAGGCCTCGTCCTACA TGAAT	216	59.8	46.3

3.6. Discussion

Morphometry is one of the cheap and best methods to identify an object when it is intact and can be done at the field itself and by ground level staffs. Morphometry of rhinoceros horns of different species have been studied by various scientists (Groves 1971; Kingdon 1979; Dinerstein 2003; Hieronymus *et al.* 2006). Attempts were made to characterize Greater Indian one horn rhinoceros based on morphometry with illustrated figures to explain and differentiate it from fake rhinoceros horn (Fig. 3.1, 3.2, 3.3)

The colour of African rhinoceros horn was dark grey, which was also similar to observation by Groves (1971). It is convincing that the horn is darker in adults than juvenile according to his observations. He also adds that the stem is darker than base, darker in the Asian than African rhinoceros. Absence of bony core in rhinoceros horn was noted in the study, which is also mentioned by Hieronymus *et al.* (2006). Mean weight and standard deviation of young and adult rhinoceros horns was 214.47±143.46 and 849.38±236.87 respectively.

Fibers and tubercles on the dorsal surface under low magnification were observed in this study and these may be easily used for identification purpose (Fig. 3.2). Distinct furrow at the anterior portion of dorsal surface (Fig. 3.2d) is an important feature which, was also mentioned by Dinerstein (2003) and might help in holding the rhinoceros horn on the snout. In some samples, tip of the horn was either dull or shining (Fig. 3.2e). Bulge, concavity and the porous ventral surface are characteristic of the rhinoceros horns (Fig. 3.3a,b&c). These characteristics were also noticed in the rhinoceros horn from zoo. Ventral surface is much more useful to distinguish rhinoceros horn, as in zoo rhinoceros horns, which was used, dorsal stem was not developed. Thus, for zoo rhinoceros horn ventral surface will be of utmost important for characterization.

Dinerstein (2003), reports that in young adult, horn is intact, with no erosion near base and short i.e. 15 cm for females and 18 cm long for males. According to him the horn of intermediate age adults shows moderate erosion at the base and is 20-28 cm long in females and 20-30 cm long in males and mostly intact and circumference at the base of the horn is 48-54 cm for males. In old adults, the horn is either long (20-33cm) or broken and heavily worn and eroded, often with deep anterior and occasionally with posterior grooves. In males horn, base is large in circumference (55-95cm), commonly with signs of breakage and subsequent re-growth (Dinerstein 2003). We did not try to age the rhinoceros horn but, the circumference and the erosion noticed in rhinoceros horn under this study were similar to Dinerstein (2003). In the zoo samples, the basal circumference was more, this might be due to low growth of stem. Angle of the rhinoceros horns was measured and it was ranged between 115 and 145^0 (Table 3.3). Circumference/weight

was calculated to minimize the age effect and this can be used to differentiate fake horns from rhinoceros horn (0.6-2.6 mm/g) (Fig. 3.3b). Rhinoceros horns can be differentiate from buffalo horn by measuring density, as both have different densities 7.49 g/cm^3 and 4.54 g/cm^3 respectively. Mann Whitney U test for densities were 0.0001 and Asymptotic significance (2-tailed) were 0.025. Kingdon (1979), used morphometric characteristic to differentiate horn of the female from males of similar age group.

Two functions derived using seven quantitative variables could absolutely differentiate the rhinoceros horn from wild and zoo as well as fake using Discriminant Function Analysis (DFA). First function could itself explain 96.9% variability (Table 3.6&3.7). The differentiation of the zoo rhinoceros horn is mainly due to the absence of stem thus, we need to check it using more samples of rhinoceros horn from zoo. Canonical Discriminant function could cluster separately, rhinoceros horn from wild, zoo and fake (Fig. 3.5). DFA will be very important in future for identification of horn of Greater one horned rhinoceros and also the scoring equation can be used to distinguish it from fake horns.

When finished product of rhinoceros horn is seized, it is sometime difficult to characterize the rhinoceros horn through morphometry. Hieronymus *et al.* (2006) has done an excellent study based on microscopy and x-ray tomography, for ultra structure of the horn under microscope and ultraviolet light. Few studies are on scanning electron micrography of rhinoceros horn (Lynch *et al.* 1973; Hsieh *et al.* 2001). Hsieh *et al.* (2003) identified sculptors made up of rhinoceros horn using SEM. Scanning microscopy was used to study both dorsal and ventral surface and the structure noticed were distinct that is presence of compact hair growth dorsally (Fig. 3.6a) and presence of uniformly placed pores having mean diameter of 320 µm and these ranged from 160 to 400 µm on the ventral surface (Fig. 3.7a). Hair like structure was also noticed by Hsieh *et al.* 2001. Presence of sub-pores having diameter 9.39 µm and these ranged from 5.56 to 10.11 µm within a pore is very remarkable and can be used for identification of rhinoceros horn (Fig. 3.7c) from others. These pores seem to be similar to the tubules examined by Hieronymus *et al.* (2006). Attempts were made to identify three suspected rhinoceros

horn using SEM and could successfully differentiated them based on above unique surface morphology of rhinoceros horn. These samples were actually fake. The buffalo horn micrographs of dorsal surface does neither indicates presence of hairs (Fig. 3.6b) nor pores were present on the ventral surface instead there were presence of twisted fibers which had dark zone in the centre (Fig. 3.7e&f).

It appears that there is no study available on X-ray diffraction of rhinoceros horn. In this first attempt, X-ray diffraction was used to analyze rhinoceros horn samples and characteristic minute peaks at 26^0, 43^0 and 50^0 were identified (Fig. 3.8a), which were not noticed in X-ray diffractogram of buffalo horn (Fig. 3.8b). Also background intensity of rhinoceros horn was higher as compared to buffalo horn. Two suspected cases of rhinoceros horn were also analyzed using XRD and compared with the reference diffractogram. Both the samples one of them in powder form, found to be fake (Fig. 3.8c,d&e). Thus, these characteristic peaks can be used for identification purpose and the specificity of the diffraction pattern may distinguish the rhinoceros horn efficiently from other horns.

Research in forensic sciences has not used, differential thermal analysis (DTA) much, with few exceptions, concerning explosives and the crimnalistics research of Rynearson and De Haan. Villanueva *et al.* (1976) used DTA and thermogravimetric analysis (TGA) to show the mineralization process in postmortem bones. Exothermic or endothermic peaks can be noted on a differential thermogram. The shape and size of the peak provide information about the nature of the test sample (Chitwal and Anand 1996). There are various application of DTA in physical chemistry including reaction, specific heat, thermal diffusivity and miscellaneous applications, analytical chemistry including identification of substances, identification of products, melting points, quantitative analysis, quality control, inorganic chemistry and organic chemistry (Chitwal and Anand 1996). But, no study has so far been recorded for rhinoceros horn based on thermogravimetry. A characteristic exothermic peak in thermograph of rhinoceros horn in between 200 and 400 ^{0}C would be useful in identification of rhinoceros horn products

(Fig. 3.9a). Further, the consistency in trend of weight loss and water loss with constant increase in temperature may be used as a specific features (Fig. 3.9 c&d). Other horns thermographs need to be checked for the pattern of weight loss and determine that those are different from the characteristic of rhinoceros horn.

The chemical composition reflects what the animals have eaten throughout their lives, and the chemical properties of their food are absorbed into the horn through digestive processes (Amin *et al.*, 2003). The qualitative elemental fluorescence X-ray scan obtained from rhinoceros horn reveal that the intensity (KCps) of iron (Fe) was highest where as intensity of magnesium (Mg) was lowest among of the fourteen elements analyzed (Fig. 3.10).

ICP-MS revealed that out of the elements analyzed in Asian rhinoceros horn, calcium (Ca) was the major component (67%), followed by iron (Fe) (12%), sodium (Na) (10%), magnesium (Mg) and potassium (K) (4%), cobalt (Co) (2%) and zinc (Zn) (1%) with other elements in traces (Fig. 3.11a&b, Table 3.10). Three elements viz. Pb, U and Cd were however not detected. The result of the study indicates that calcium is more in rhinoceros horn and is supported by the findings that the non-ruminant ungulate equids, tapirs, rhinoceros and elephants absorb a larger proportion of dietary calcium than ruminants do (Schryver 1983) as feces of equids, tapirs, rhinoceros and elephants had a lower calcium concentration and a lower Ca/P ratio than feces of ruminants when the animals were fed diets of equivalent calcium content. Hieronymus *et al.* (2006) infers that the rhinoceros horn is nature's composite material and is composed of calcium and melanin and has higher concentration in middle of the horn. Hsieh *et al.* (2003) results indicated that sculptors made of rhinoceros horn were made up of hair fibers and the major element detected was sulphur rather than phosphous and calcium.

However, mean concentration of four elements Ca, Na, V and Pb were different in wild and the zoo rhinoceros horns. The rhinoceros horns from zoo have higher concentrations of all four elements and the difference was almost thrice except calcium (Ca). The difference in calcium (Ca) and sodium (Na) concentrations may be due to

differences in feeding habits whereas high vanadium (V) and lead (Pb) concentrations in horns of zoo animals reveals that the atmosphere of in wild is less polluted than zoo in metropolitan city (Fig. 4.9d&e). The amount of calcium was variable and it might be due to age or physiological condition of individual. More samples from zoo should be checked to see if concentrations of these elements are high consistently than these may be used in distinguishing zoo rhinoceros horn from the wild rhinoceros horn. According to Thronton (2002) the chemistry of food varies in response to an area's underlying geology; geomorphologic history, and climate. Thronton (2002) reports about the lack of data of the mineral requirements of wildlife species prevented an assessment of the risk of deficiencies in these species, including rhinoceros. Several authors (Lee-Thorp *et al.* 1992; Hall-Martin *et al.* 1993; Hart *et al.* 1994; Emslie *et al.* 2001) have used chemical techniques to differentiate species and geographical source of the African rhinoceros horn based on the variation in the concentration of elements and isotopes and their ratios. Amin *et al.* (2003) used three different chemical techniques, and Discriminant Function Analysis (DFA) to distinguish between species and origin of illegally traded or confiscated African rhinoceros horn. Thus, chemical technique can be used to differentiate rhinoceros horns of different regions as well as from other horns.

Number of protein bands and their positions were variable in rhinoceros and buffalo horns. Therefore, these bands may be used as distinguishing feature between these two species. It is also expected that differences are bound to occur in horns of other species. Slight differences in position of protein bands were also visible between rhinoceros horn from wild and zoo. Therefore, the characteristic protein bands position may serve for characterizing rhinoceros horn and differentiating it from buffalo horn, as well as from fake rhinoceros horn. No bands were noticed in fake rhinoceros horn (Fig. 3.12a). Cluster analysis (Ward's method) clearly differentiates rhinoceros horn and buffalo horn based on presence of protein bands at different positions (Fig. 3.12b, Table 3.11&3.12). Hieronymus *et al.* (2006) concludes that rhinoceros horn is composed of keratin. Butler *et al.* (1990) used isoelectric focusing to identify rhinoceros keratins and their basis of investigation was based on polymorphic nature of keratins, which can be used for species identification. They could differentiate rhinoceros horn from other horn

and antler using isoelectric bands and silver staining. They hope that better extraction methods as well as separation procedures can be developed. Block (1939) concludes that the hardness of rhinoceros horn is due to presence of high amount of tyrosine and added that it has less amount of cystein. Hunga and Yu, 1997 used capillary isoelectric focusing (CIEF) method to differentiate rhinoceros and buffalo horn and the result demonstrated that α-keratins in the horns of buffalo and rhinoceros might have their unique isoelectric focusing profiles.

Most of the molecular DNA study is for phylogenetic purpose (Tougard *et al.* 2001, Hsing *et al.* 2003). DNA was successfully extracted and amplified (Fig. 3.13a &b). Sequence of rhinoceros horn was different from buffalo horn. Hsing *et al.* (2003) has also mentioned that the sequence of rhinoceros horn of various species is different and these may help in categorizing the rhinoceros horns of different species. He had used only one sample of Greater Indian one horn rhinoceros which can be supplemented with our study. They could sequence 402 bp of rhinoceros horn and reported that black rhinoceros was at greatest distance from Indian rhinoceros. Their study supports morphological hypothesis of phylogenetic study. Conversely, Tougard *et al.* (2001) used cytochrome b and 12S rRNA sequence to corroborate the geographical closeness hypothesis, which clusters the African species with the other two Asian species. Kapur *et al.* (2002) identified pSG5 clone of 688 bp band specific to *Rhinoceros unicornis* which had repeats of "GT" using mini-satellite associated sequence amplification (MASA). Thus, this genetic marker will help in species identification.

DNA was successfully extracted, and amplified using cytochrome b and 16 S rRNA gene. Sequences of cytochrome b region for *Rhinoceros unicornis*, *Bubalus bubalis* and Bos *taurus* were aligned using Bioedit software. Analysis of sequences revealed that more then 24.65% (90 sites) sites were polymorphic of the 365bp of cytochrome b alignment. Restriction enzymes which cut the cytochrome b gene fragment at different sites in rhinoceros and buffalo are listed in Table 3.12. These may be used to differentiate these species from one another. Number of restriction enzymes which cut

DNA fragments of *Rhinoceros unicornis*, *Bos taurus* and *Bubalus bubalis* are 5, 15 and 49 respectively. None of the restriction enzyme is common to all these three species. Primers were designed of 210-250 bp to be used for all the three species will help in differentiating these species using DNA sequencing (Table 3.13).

Thus, all the techniques used indicate that Great Indian rhinoceros horn has unique signatures. These unique signatures may serve as tools to identify intact and powdered rhinoceros horn, and sculptors, contributing in conservation of Greater Indian one horn rhinoceros species. Therefore, the hypothesis that morphological, crystallographic (XRD & XRF) and DNA characteristics of Great Indian one horned rhinoceros are species specific may be accepted.

Table 3.14. Ranking of different methods to be used for characterization of rhinoceros horn.

Types of samples	Ranking for use of different techniques							
	Morphometry	**SEM**	**XRD**	**TGA**	**XRF**	**ICP-MS**	**Protein profile**	**DNA**
Complete	II	I	III	VI	VIII	VII	IV	V
Pieces	-	I	II	V	VII	VI	III	IV
Powder	-	-	I	IV	VI	V	II	III

Ranking order is in decreasing order: I= Best

3.7. References

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CHAPTER 4

Characterization of antler

4.1. Introduction

Antlers are unique characteristic of family Cervidae in deer species and fully regenerate each year in relation to their sexual cycles (Chapman 1975; Goss 1983; Lincoln 1985; Bubenik 1990). It is mostly secondary sexual characteristic of males except in genera *Rangifer*, where it is found in both sexes (Kiltie 1985; Bubenik 1990). In males it is principally used as weapons in intraspecific fighting or possibly as organs displaying strength (Kitchener 1991; Andersson 1994). These are bony outgrowth of the cranial frontal bones (Bubenik 1966; Goss 1983), and differs from keratinous horn of the Bovidae in that horns are not cast and re-grown annually, and the most active zone of cell division is at the base of the horn, whereas in antlers growth occurs at the tip (Goss 1983). Antlers are branched and solid (Hanfee 1998), whereas horns are un-branched and hollow.

Antlers have been studied in detail on taxonomical, morphological, endocrinal and genetical aspects and well documented by various scientists throughout the world (Geist 1966; Darwin 1871; Clutton-brock 1982; Packer 1983; Berger and Cunnigham 1995; Emlen 2001). Bubenik (1966) studied phylogenetic aspect of bovid, cervidae and giraffid and reported that horns and antlers both are non-homologous in their development. Bubenik (1968) reported that antlers are useful in visual characterization, distinguishing sex, species and individual. According to him the difference in shape and colouration of antlers are related to the biome type. Antler characteristics have been used to determine the age class of male swamp deer and other deer species through appearance of small bumps, spikes, length of the antler and distance from the pedicle to the tip (Martin 1975).

Graf and Nichols (1966) noted that antlers circumference and beam length is roughly related to the size of the male deer. But, they further reports that food quality and quantity, and heredity can influence antler size and could upset the relationship

with body size. Scribner and Smith (1990) reported that antler size and growth in different individual varies in relation to age, level of heterozygosity, body size, nutritional condition and body condition. They further added that environmental condition has significant effect on antler size. A similar observation that antlers are more responsive to environmental stress than other morphological features was also noted by Ashley *et al.* (1998). This makes them indicator of physical health of population (Severinghaus and Moen 1983; Ramussen 1985). Evans *et al.* (1999) reported that antler size is correlated to the age, health and genetics of male. Caro *et al.* (2003) studied the correlations of antler shape in cervids and reported that the antler shape does not have any correlation with environmental factors and female selection, instead it is weakly correlated with body size and intraspecific fighting behaviour and the extent which individuals are expected to fight, for mating.

Dziedzic (1992) studied length and frequency of occurrence of tines in roe deer antlers reported that the highest percentage of tine occurrence took place in 6 year old animals and the lowest in 2 year old male. He observed difference in occurrence of front and back tines in different age groups. In 2 year old animal the percentage of front tine were high which decreased with age in the group of animal above 7 year. Graf and Nichols (1966) also reported a similar observation in *Axis* species that the number of tines decreases in older males. Caro *et al.* (2003) reported that antlers with tip facing out and with many tines are observed in large cervids and those with inward facing tips in small cervids. Raman (1998) studied the antler cycle and breeding seasonality of the chital in Southern India and reported that only adult males attained peak hard antlers during the month when most conceptions occurs which is significantly correlated with fawning. He further reports that the peak in hard antler of juvenile and yearling males occurred 2.5 to 5 months later than in the adult males.

According to Bubenik (1968), the antlers are formed by the calcification, beneath the velvet of the fast growing pre-oessous tissue known as velvet antlers. Continuous dividing velvet antlers replace dead antlers every year through shedding. Analysis of velvet antler reveled that it is composed of 54% organic substance, 34%

ash and 12% moisture (http://www.brunnerbiz.com/activelife/activelvet.html). Further detail analysis indicated 47% protein, 33% mineral, 3-4% lipid and 3% sugar. Major protein is collagen with major amino acids, glutamic acid, alanine, glycine and proline, minerals are calcium, potassium, chlorine, sulphur, phosphorous, silicon, magnesium, sodium, nitrogen, manganese, iron, copper and zinc, major lipids are gangliosides, sphingomyelins, steroids with small fraction of prostaglandins, sugar is mucopolysaccharides (http://www.brunnerbiz.com/activelife/activelvet.html).

Antlers are composed primarily of minerals, protein, carbohydrates and fatty acids (Sunwoo *et al.* 1995). Calcium and phosphorus, primarily present at hydroxyapatite ($Ca_5(PO_4)_3OH$), constitute 38% and 18% of the ash, or approximately 21% and 10% of the dry mass of the antlers, respectively (Chapman 1975). Moen *et al.* (1999) used a simulation model of energy and mineral metabolism to compare nutritional requirements for antler growth in Irish elk and moose, the largest extant cervid. They predicted that the Irish elk antlers weighing 40 kg would contain 2.1 kg nitrogen, 7.6 kg calcium and 3.8 kg phosphorus. According to this model, to grow 10 kg antlers in a 150 day period, daily deposition of calcium and phosphorus were more than 60 g and 30 g respectively during high mineralization rate of antler in mid-summer. When requirements for deposition of calcium and phosphorus in the antler can not be met by dietary intake therefore skeletal minerals are reabsorbed (Banks *et al.* 1968).

Antlers are highly sensitive to nutrition (Vogt and Schmid 1950; Goss 1983) being tissue of low growth priority. Large antlered species owing to their greater demand for nutrients and energy for reproduction and antler growth should have greater tendency to feed on forbs and leaves, because these contain more calcium, phosphate and protein (Vogt 1948). Mineral required by herbivores are derived from plant tissues but deposition of minerals in plants is limited by quality of the soils and the underlying geology of the region (Van Soest 1994).

The developing antler is composed of an aggregate of distinct cell types including fibroblasts, chondroblast, chondrocytes and osteocytes (Banks and

Newberry 1982). The tip of velvet antler has few layers of mesenchymal cells which abruptly differentiate to cartilaginous tissue, which are afterwards replaced by bone (Fenessy and Suttie 1985). Speer (1983) studied the histology of antler using polarized microscope and reported that longitudinal section showed the presence of epidermis, dermis, peristomium having coarse collagen fibers followed by fine collagen fibres and spongy cells in the center.

Antler bone is considered to be one of the toughest bones (Zioupos *et al.* 1994; Vashishth 2004). The outer cylinder of compact antler bone coupled with the spongy core imparts maximal strength to the antler (Goss 1983). In autumn the greater fluid content of antler bone endows it with maximum resistance (Chapman 1981). Zioupos *et al.* (1994) reported that crack propagation-based measure of bone toughness demonstrated that cortical bone obtained from antlers of red deer is twice as much tough as bovine cortical bone. Studies have shown that initiation toughness correlates with varying degrees of success to compositional and morphological parameters of bone including mineralization, porosity and osteonal morphology (Phelps *et al.* 2000).

Antler growth is related to general body growth (French *et al.* 1956) and guided by hormones (Bubenik 1990; Suttie and Bubenik 1992). Fennesy (1992) reported an elevated level of natural growth hormone called insulin like growth factor (IGF-1) and its receptors in the blood of deer during antler growth. Suttie and Bubenik (1992) mentioned that high androgen level is needed for initiation of pedicle growth and antler mineralization; where as lowering of androgen level is responsible for antler casting. Experimental data indicates that estradiol may be responsible for antler ossification and dihydrotestosterone (DHT) for desiccation and death of the velvet (Fenessy and Suttie 1985). Suttie and Bubenik (1992) reports testosterone can be converted into estradiol or to dihydrotestosterone.

Suttie (1990) reported that the antler size and shape are determined genetically and control by nervous system. Wemmer (1987) reported that deer shows considerable interspecific variation in chromosome number. Taylor *et al.* (1969) and Neitzel (1982) have studied on the genetics of cervid. Cronin (1991) studied on the

population variation of cervids and reported that unlike other large mammals, they show high degree of genetic variation within population. DNA can be extracted from antler to develop a simple genetic species test using nuclear or mt-DNA. Now many microsatellite and DNA markers are available for cervids (Anon. 1999).

There are nine species of deer in India viz. Kashmir stag (*Cervus elaphus*), mouse deer (*Tragulus meminna*), musk deer (*Moschus moschiferus*), swamp deer (*Cervus duvaucelii),* thamin (*Cervus eldi*), barking deer (*Muntiacus muntjak*), chital (*Axis axis*), hog deer (*Axis porcinus*) and sambar (*Cervus unicolor*). Antlers of chital, sambar and swamp deer are widely traded (Hanfee 1998). Previously the export of shed antlers and peacock feathers was allowed in India. Menon *et al.* (1994) reported that the antlers export from India was mostly to the Europe and East Asia. In 1993 antlers export from India to Taiwan alone was more than US $ 100,000 (Fitzgerald 1989). New Zealand exports nearly 450 tones velvet antler per year and is the largest supplier followed by China (http://www.american.edu/projects/mandala/TED/deer.htm). Korea and Hong Kong are the largest importers of velvet antler but much of this is assumed to be re-exported to the west in either powder or some other processed form (http://www.american.edu/ projects/mandala/TED/deer.htm). Antlers are used either as trophies (shields) for decoration or to make cutlery handles, pistol butts, show pieces, hanger, buttons and furniture (Menon *et al.* 1994). It is also used in traditional oriental medicines (Hanfee 1998; Sim and Sunwoo 2001) for treatment of anemia, arthritis, impotence, dizziness, insomania, amnesia, wound, pain, epilepsy, baldness, pimples, lice, toothaches, febrile infections and snakebites in different countries (Kong and Ko 1987; Yoon 1989). The problem of illegal trade of antlers is mainly due to the misdeclaration of its quantity i.e. in place of 100 metric tonnes allowed consignment of antlers, 150 or 200 metric tonnes goes out (Aziz 1996).

Visualizing the current status of some of the deer species, trade of antler in India has been banned recently ((Dey 1996). To provide legal protection to deer and other related species accordingly brow-antlered deer (*Cervus elidii*) and swamp deer (*C. duvauceli*) were included in Schedule I, whereas barking deer (*Muntiacus muntjak*), chital (*Axis axis*), hog deer (*Axis procinus*) and sambar (*C. unicolor*) were

in schedule III of *Wildlife (Protection) Act, 1972* (Hanfee 1998). Mouse deer and musk deer possess only canines and antler is absent in both the deer species (Whitehead 1993).

To strengthen the legal measures to protect Indian deer species, identification of antlers in complete or pieces or powder form is very important. Very few scientific studies deal with identification of antlers belonging to different species (Caro *et al.* 2003). Thus, present study is aimed to identify species from antlers using various techniques. Morphological features of antlers in artiodactyls often used as a diagnostic tool to identify species (Caro *et al.* 2003). Although, morphometry is very good technique to identify antlers but as malformation in antlers is common (Graf and Nichols 1966) and antlers are seized either in complete form or in small pieces (Anon. 1997) or powder form, therefore, identification through morphometry becomes difficult for deformed antlers. Hence in this study different techniques viz. morphometry, analytical and molecular techniques have been used. This study will help managers, scientists and enforcement agencies to identify species from parts and products of antler.

4.2. Objective

Determine morphological, crystallographic (XRD & XRF) and DNA characteristics of antler of chital, sambar and swamp deer species.

4.3. Hypothesis

Morphological, crystallographic (XRD & XRF) and DNA characteristics of antler of chital, sambar and swamp deer are species specific.

4.4. Materials and methods

Antlers used in the study were from the collection of teaching and forensic laboratories, Wildlife Institute of India, Dehradun, some antlers were measured at Dudhwa and Corbett National Parks, and some antlers were procured from Assam Forest Department. Attempts were made to characterize antler of different deer

species using structural patterns, chemical constituents and DNA sequences. Table 4.1 indicates about the methods used and number of samples analyzed.

Table 4.1. Different methods used and number of antler samples analyzed for different species.

Techniques	Number of samples				
	Chital	Sambar	Hog deer*	Swamp deer	Barking deer*
Morphometry	49	31	31	26	8
SEM	6	4	3	6	1
XRD	5	7	5	4	1
XRF	1	1	1	1	1
ICP-MS	7	4	4	4	1
TGA	1	2	1	2	1
DNA	1	1	0	1	0

* Used for comparison with other species.

4.4.1. Morphometric and histological differentiation of antler

Species identification of antler of Cervidae was based on morphology depending on the number of branches, their curvature, length, circumference, number of grooves, colour, texture and branching pattern. Datasheet was prepared and accordingly all measurements of antlers of different species were recorded. Total 22 variables were measured other than angles including four qualitative and 18 quantitative variables. All quantitative measurements were mainly recorded in millimeter (mm) using vernier caliper and where it was not possible to measure using vernier caliper, it was measured with the help of thread and scale (Table 4.2).

Table 4.2. Quantitative measurements recorded for antlers.

Sl. No.	Variables selected for measurements
1	Circumference below the base
2	Circumference above the base
3	Circumference at 5 cm above the base
4	Circumference at 5 cm from branching on the main branch
5	Circumference at 5 cm from branching on the I^{st} tint
6	Circumference at 5 cm from tip of the I^{st} tint
7	Length from base to I^{st} branching
8	Length from base to tip of main branch
9	Length from base to tip of I^{st} tint
10	Length of I^{st} tint
11	Circumference at 5 cm from branching on the II^{nd} tint
12	Circumference at 5 cm from tip of the II^{nd} tint
13	Length from base to II^{nd} branching
14	Length from base to tip of II^{nd} tint
15	Length of II^{nd} tint
16	Angle at first branching
17	Angle at second branching
18	Grooves

Texture, shed or unshed, branching pattern, curvature, number of tines and presence of grooves in antlers were recorded visually. Antler was examined for hard as well as for velvet antlers. Few morphologically distinct features were noted during physical examination of antlers. Angles indicated in Fig.4.1 were drawn on sheet of paper and was measured using a protector. Histograms of the angle measurements for antlers of various deer species were plotted. Discriminant functional analysis of the quantitative measurements was performed to differentiate antlers of various species. Cross-sections of antler of various species were observed to distinguish among them.

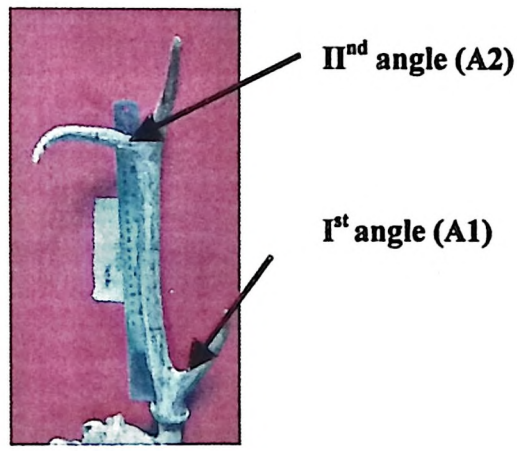


Figure 4.1. Angles used for characterization of antler.

4.4.2. Scanning electron microscopy

Antlers of various species were examined under scanning electron microscope. The number of antlers of different species analyzed are given in Table 4.1. Three small pieces of 5mm width, 5 mm length and 2 mm thickness, from outer, cortex and core region were cut for antler of each species. The preferred portion was the main beam after first branching. The outer, cortex and core area were polished to remove any induced marks during cutting and all three pieces were cleaned properly using alcohol. Samples were prepared by cleaning, mounting and coating with gold for better conductivity (Goldstein *et al.*, 1981) to facilitate their examination under the Scanning Microscope (PSEM 515, Philips, Holland). The require micrographs were saved using software Digital Image Scanning System (DISS 5, Point Electronics, Germany). The micrographs obtained of antlers of various deer species were compared at all the above mentioned three portions to note the species specific characteristics.

4.4.3. X-ray diffraction

Powder of the antlers of chital, sambar, swamp deer, hog deer and barking deer of particle of 300 mesh sizes were prepared for examining under the diffractrometer (PANalytical, Philips, Holland) to note the differences in the diffractogram pattern of antler of different species. The number of antlers of different species analyzed is furnished in Table 4.1. Presence and absence of peaks were noted. For hexagonal crystal, cell parameter a = b hence, a & c were calculated manually using formula $1/d^2 = 4/3\ (h^2+hk+k^2)/a^2 + l2/c^2$, where, d = Diffraction lattice; h,k,l = miller indices; a & c = cell parameter and Cell volume was also calculated manually using formula $V = 0.866\ a^2\ c$, where, V = Cell volume. Further cell parameter and Cell volume were compared (Cullity 1978).

4.4.4. Thermo gravimetric analysis

Small pieces of one or two antlers of each species were cut, cleaned, further dried in desiccators and ground. Approximately 10 mg of powdered samples were analyzed under Perkin Elmer thermo analyzer using alumina powder as reference in nitrogen atmosphere 200 ml/min. Temperature was uniformly increased at the rate of 10 ^{0}C/min from 20^{0}C to 1400 ^{0}C. Thermograph and data were generated using Pyris Diamond software. Thermographs were compared and further the data were analyzed to check the loss of water and weight in antler of various species.

4.4.5. Elemental analysis

4.4.5.1. X-ray fluorescence

An antler of each species was analyzed through X-ray fluorescence. Powder of antlers was used and pellets were prepared to examine under the fluorescence spectrometer (Siemens SRS 3000, Varian, Australia), to note different intensity patterns among antlers of different species. Intensities (KCps) of 16 elements were recorded using three crystals detectors LiF 200, Ge and OVO55.

4.4.5.2. Inductively coupled plasma-mass spectrometry (ICP-MS)

Further Inductively Coupled Plasma Analysis – Mass Spectrometry was used to know the quantity of different elements present. One to seven antler of each species were analyzed. The ground samples of 0.1 gm were acid digested using standard

protocol and analyzed using Perkin-Elmer SCIEX ICP-Mass Spectrometer model ELAN DRC-e, USA. Later the mean value, standard deviation and range were recorded for antlers of different species.

4.4.6. DNA analysis

Attempts were made to extract DNA from four target regions viz. basal portion, outer, cortex and core (Fig.4.2). Powder were scraped from all regions and crushed with the help of liquid nitrogen for easy DNA isolation. These crushed powder were digested using digestion buffer (10mM Tris-HCl, 10mM NaCl, 2% SDS, 0.39mM DTT and 200mg/ml proteinase K) and kept overnight at 56 °C.

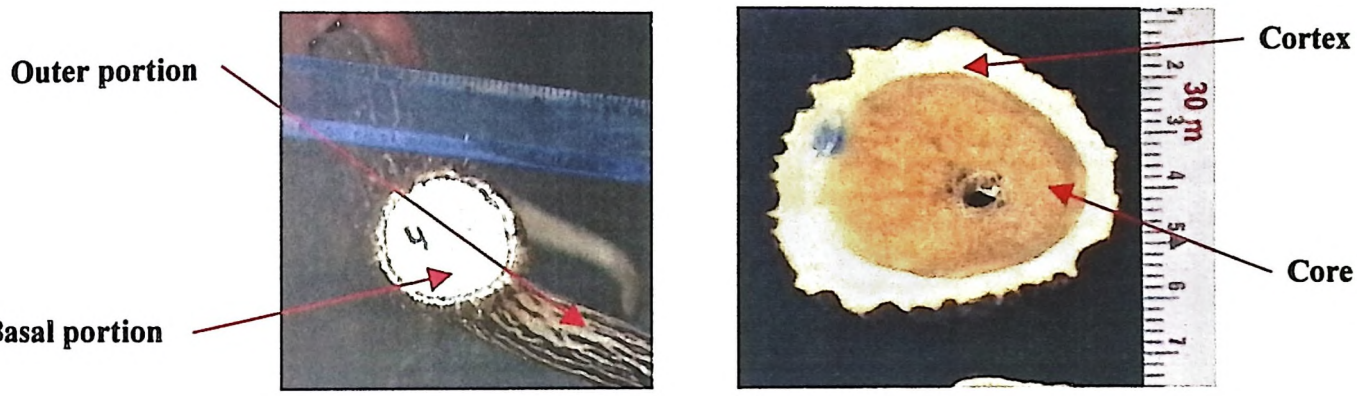


Figure 4.2 Target regions selected for DNA extraction from antler.

Three methods were used to extract DNA from samples of different target regions viz. (1) Phenol Chloroform Isoamyl alcohol treatment and alcohol precipitation (Sambrook *et al.*2001). (2) Commercially available Gene Clean Kit for Ancient Bone DNA (Bio 101® Systems), Q-BIOgene, USA (3) Sodium Acetate Precipitation Method (Cattaneo *et al.* 1995). Protocols as described by the manufacturer were followed. 8 µl extracted DNA was resolved on 0.8% gel, and photographed (UVP Bio-imaging System, Cambridge, U.K.) under UV light. Cytochrome b gene is widely analyzed for species-specific identification, phylogenetic and forensic investigations (Kocher *et al.* 1989; Hsieh *et al.* 2001; Wan and Fang 2003). As the DNA yield was low thus, partial fragments of cytochrome b gene 381 bp (Kocher *et al.* 1989) and gene of 186 bp (Farell *et al.* 2000) of mitochondrial DNA were chosen for species identification.

Primer A (381 bp) for partial fragment of cytochrome b gene:

Forward, 5'- CCATCCAACATCTCAGCATGATGAAA-3'

Reverse, 5'-GCCCCTCAGAATGATATTTGTCCTCA-3', and

Primer B (186 bp) for partial fragment of cytochrome b gene:

Forward, 5'- AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3' and

Reverse, 5'- TATTCTTTATCTGCCTATACATACACG-3'.

PCR amplification reaction was carried out in a volume of 50 µl containing 1x- PCR Buffer (50mM KCl, 10mM Tris HCl (pH 8.8), 0.08% Nomidet P40), 1.5mM $MgCl_2$, 0.5 mM dNTPs, 0.2 µM of each primer, 0.1mg/ml BSA, 1U Taq polymerase, and 50-100ηg of DNA template. The reaction mixture was initially heat denatured at 94^0C for 10 min followed by 40 cycles of denaturation at 94^0C for 1 min, annealing was similar for both primer at 47^0C for 45 sec., and extension at 72^0 C for 1 min, with a extension step at 72^0C for 10 min. 8 µl of amplification products was resolved on a 2% agarose gel in 1xTAE buffer, stained with ethidium bromide and photographed under UV light. Later, amplified PCR products were purified using QIAquick® PCR Purification Kit (QIAGEN, Germany). The DNA sequences of three species namely chital, sambar and swamp deer were downloaded from NCBI. Alignment for checking polymorphic region was performed using software Bioedit. Restriction enzyme sites were determined using software Web cutter and Primer was developed using Primer 3.

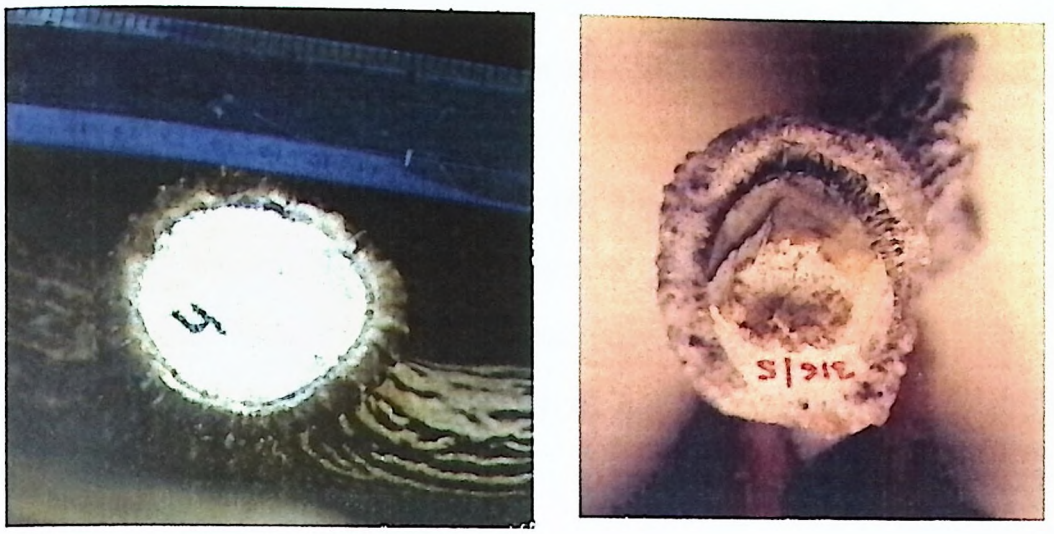
Statistical analysis used was mean, histograms and standard deviation for various parameters and Discriminant Function Analysis was carried out for quantitative measurements of antlers.

4.5. Results

4.5.1. Morphometric and cross-sectional differentiation

4.5.1.1. Morphological features

Antlers of major five species mainly found in trade were of chital (*Axis axis*), sambar (Cervus unicolor), swamp deer (*Cervus duvauceli*), barking deer (*Muntiacus muntjak)* and hog deer (*Axis porcinus*). Antlers of these species when intact were differentiated based on number of tines, branching patterns, curvature, and roughness. Shed antler did not have any cut mark and had rough bony surface on underneath the basal portion (Fig. 4.3). In unshed antler cut mark was found and pedicle bone was attached to the antler (Fig. 4.3). Velvet antler had covering of skin above the hard portion, this skin covering was not noted in the hard antler (Fig. 4.4).

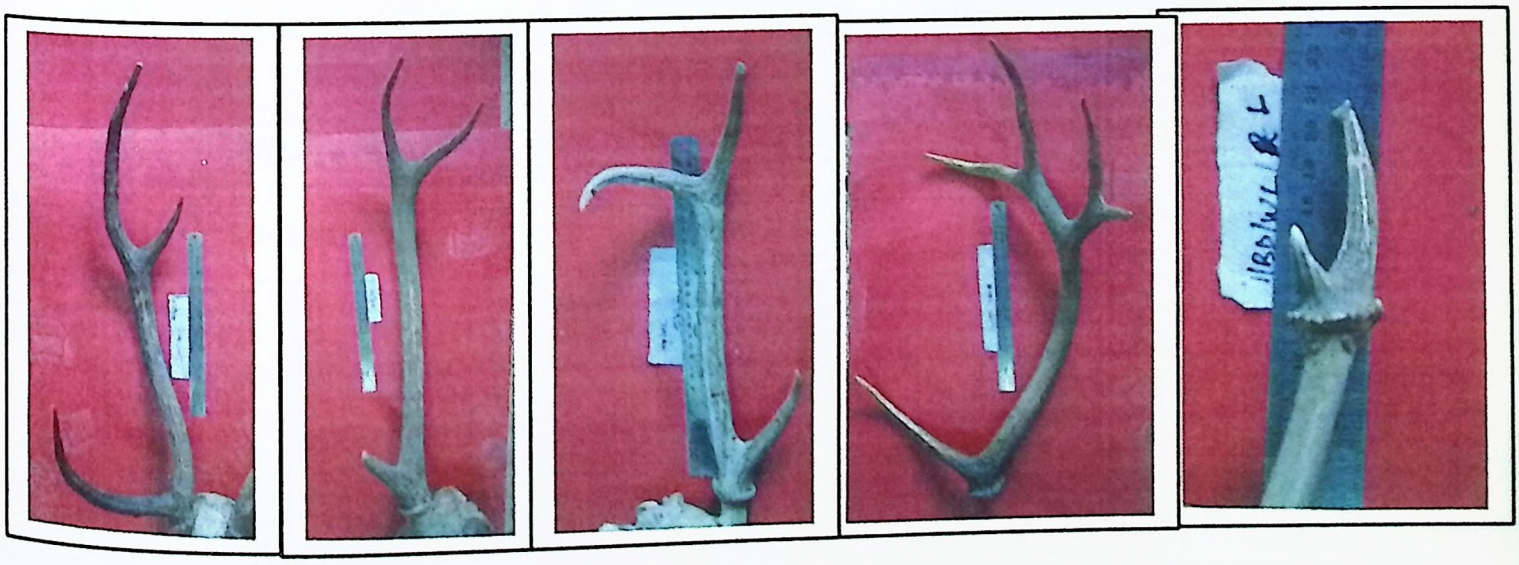


Shed antler **Unshed antler**

Figure 4.3. Differences between shed and unshed antlers.

Hard antler **Velvet antler**

Figure 4.4. Characteristics of antler.



Chital **Sambar** **Hog deer** **Swamp deer** **Barking deer**

Figure 4.5. Branching patterns of antlers in selected deer species.

The curvature of the main beam was also different in these five species (Fig. 4.5). In swamp deer antler, the main beam was curved away from the first tine, in chital the main beam was slanted towards the first tine, sambar and hog deer had almost straight main beam. Number of tines was specific to species. In swamp deer, there were five to eight tines, barking deer had only one tine and others chital, sambar and hog deer had two tines each. Number of angles depends on the presence of number of tines.

Branching pattern of barking deer antler had one tine and it was placed high on bony pedicle and the tip of the main beam was curved abruptly. Swamp deer antler had more than two tines and the antlers were of medium roughness to low roughness compared to antlers of other deer species. Branching pattern in chital, sambar, swamp deer and hog deer were different. In chital the first tine formed 'C' shaped structure which curved away from the main beam and then becomes parallel to it. In swamp deer the first branch is straight and away from the main beam. Sambar also have straight first tine near the main beam. The second tine of hog deer was pointed downward and other tines of all the other four deer and first tine of hog deer points upward. Early dichotomization of second tine was noticed in chital as compared to sambar, which bifurcates at the end.

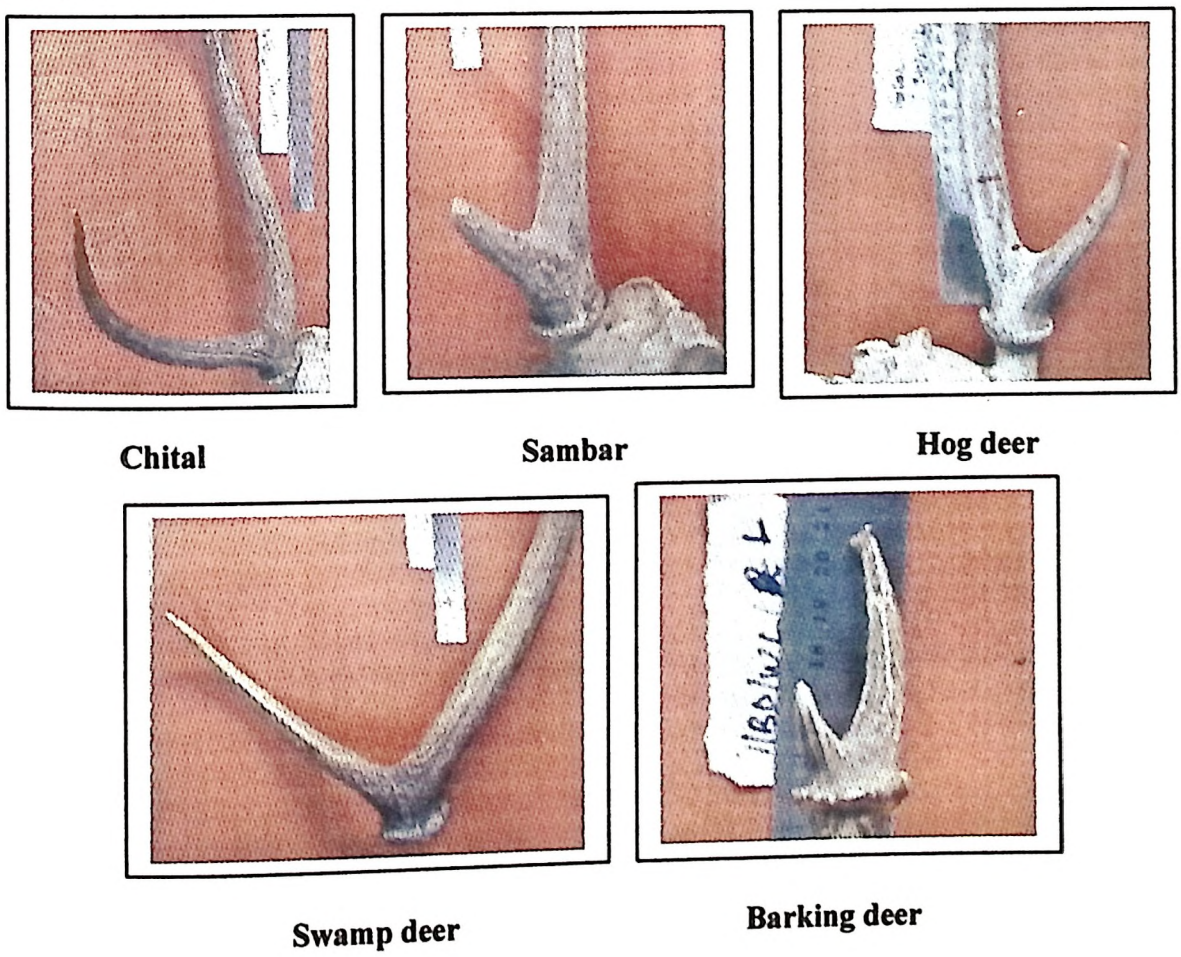


Figure 4.6. First angle of antler of different species.

The first angle in Fig. 4.6 indicates that swamp deer antler had widest angle followed by chital and sambar. In hog deer and barking deer antlers, the values of first angle were overlapping. The statistical values of first angles (A1) of antler are furnished in Table 4.2. The histograms (Fig. 4.7) indicate the mean and standard deviation of first angle of various deer species.

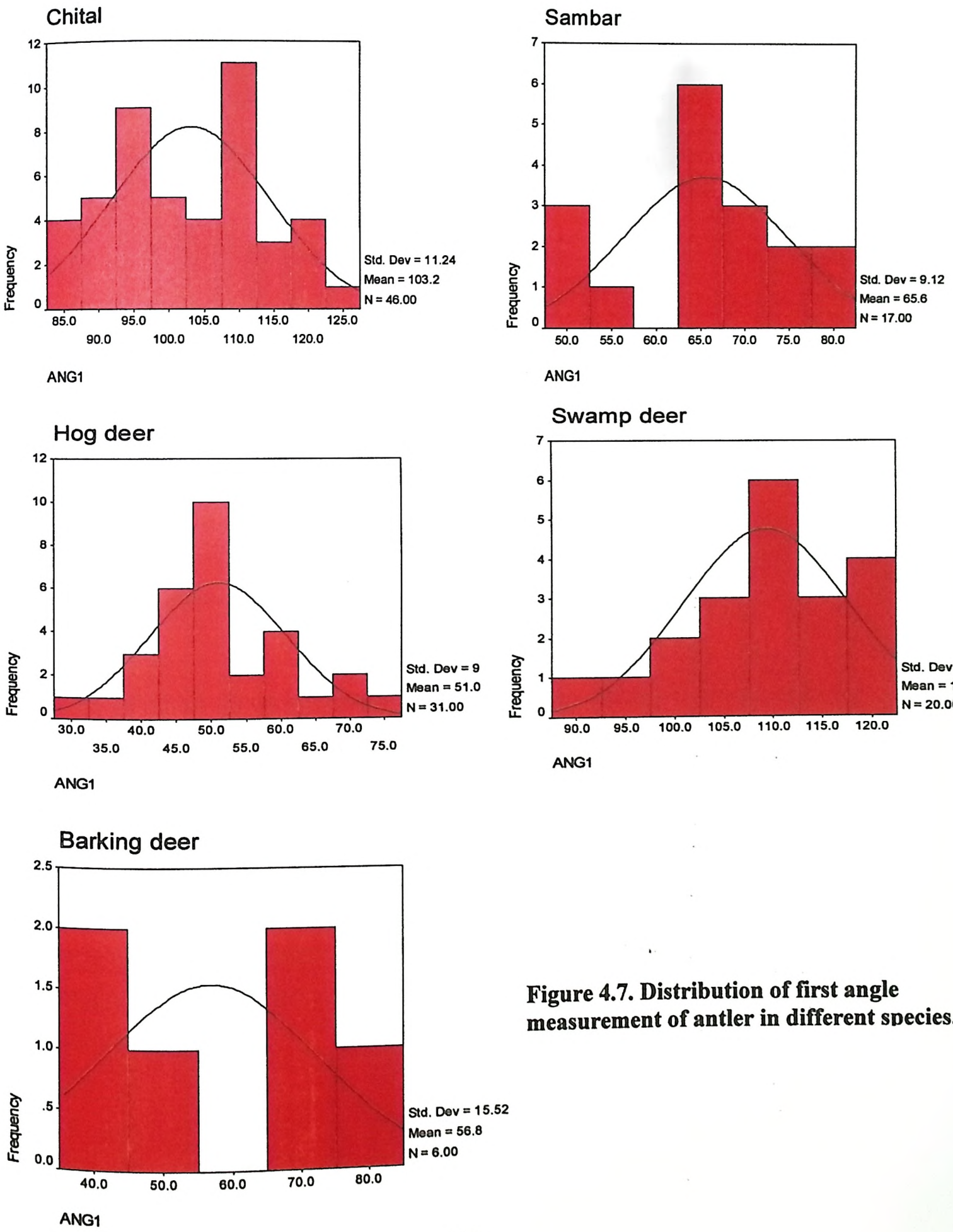
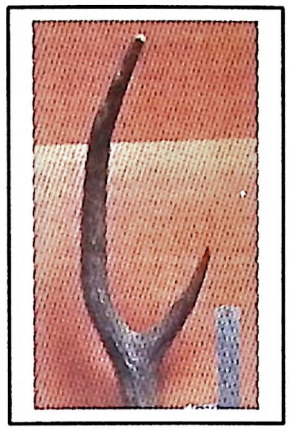
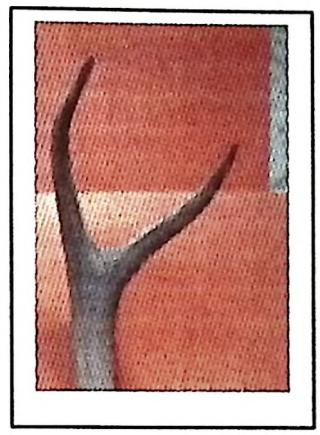


Figure 4.7. Distribution of first angle measurement of antler in different species.



Chital



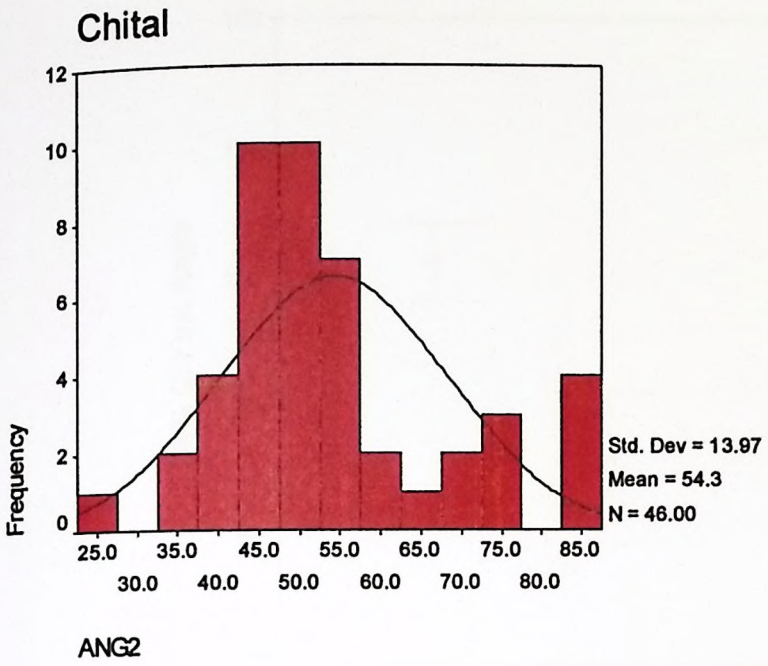
Sambar

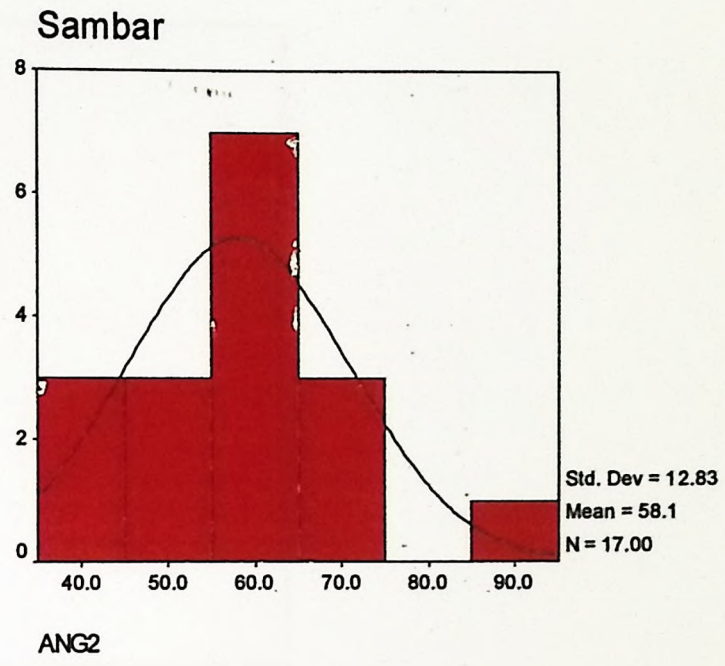
Hog deer

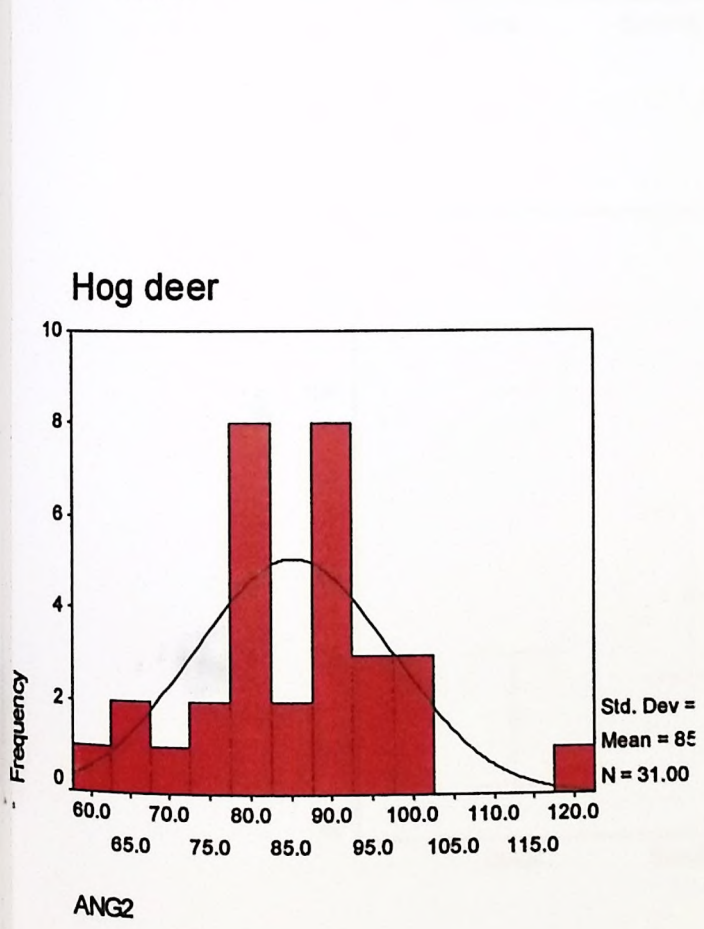
Swamp deer

Figure 4. 8. Second angle of antlers of different species.

The angle formed by second tine and the main beam was known as second angle (A2). Figure 4.8 illustrates the second angle of antlers of various species. The second angle was observed to be widest in swamp deer antler followed by hog deer, sambar and chital. As barking deer antler does not have second tines and angle. Other variations noticed were, the presence of more angles in swamp deer. The mean second angle measurement of hog deer was approximately 85^0 and formed early nearly at two-third of the main beam where as in sambar angle was formed at the end. The histograms (Fig. 4.9) show the mean and standard deviation of second angle of various deer species.







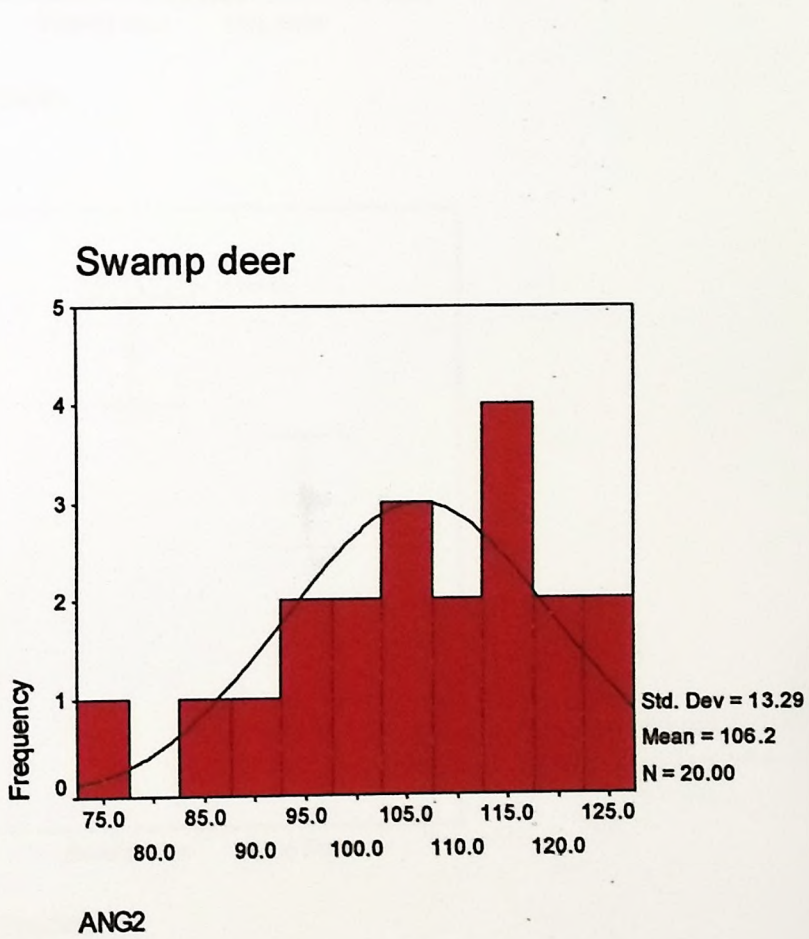
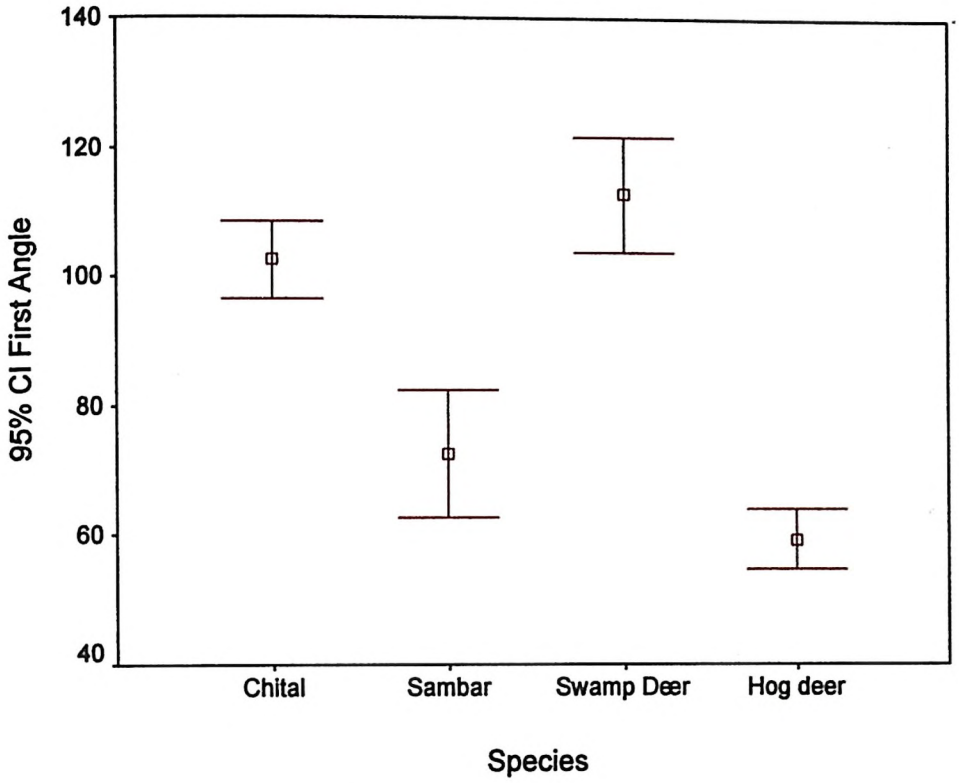


Figure 4.9. Distribution of second angle measurement of antler of different species.



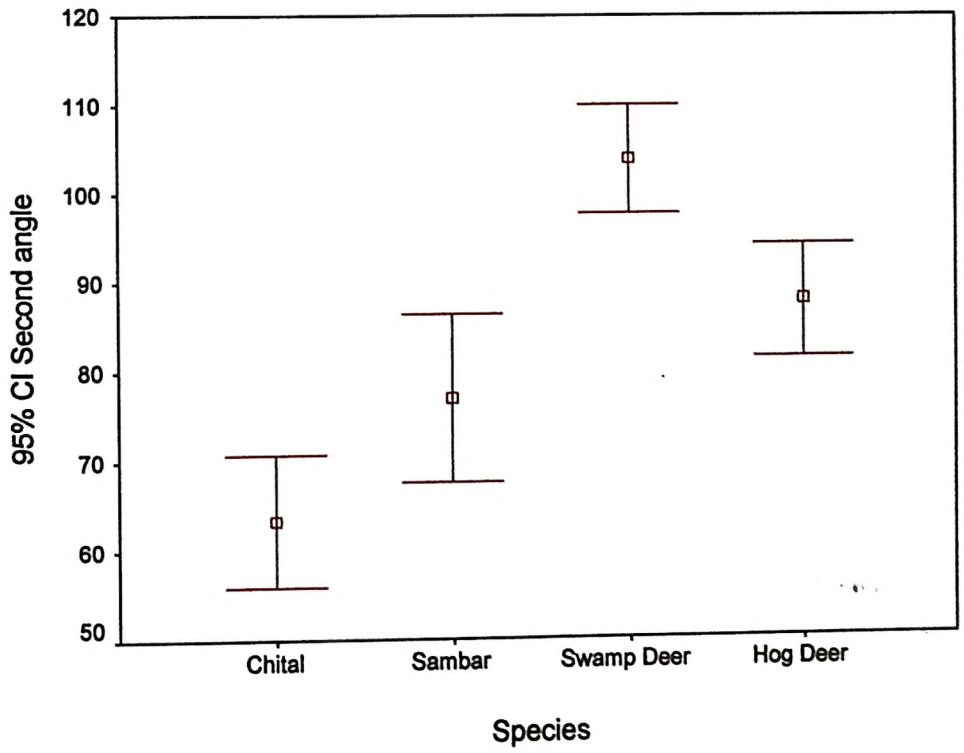


Figure 4.10. Error bars showing mean and confidence interval in both first and second tine angles.

Different confidence intervals were noticed in the first angle of all four species without any overlap (Fig. 4.10). But, the confidence intervals of second angle showed overlap in three species except swamp deer (Fig. 4.10).

Table 4.3. Mean, standard error and coefficient of variance of first and second angles of antlers of five deer species.

Species	N	A1			A2		
		Mean	SE	CV	Mean	SE	CV
Chital (*Axis axis*)	46	103.17	1.66	126.28	54.35	2.06	195.21
Sambar (*Cervus unicolor*)	17	65.59	2.21	83.26	58.12	3.11	164.49
Hog Deer (*Axis porcinus*)	31	51	1.76	96.47	85.26	2.18	186.87
Swamp Deer (*Cervus duvaceli*)	20	109.25	1.87	69.88	106.20	2.97	176.59
Barking Deer (*Muntiacus muntajac*)	6	56.83	6.34	240.97			

Table 4.3 indicates the mean, standard error and confidence interval of first and second angle measurements of the deer species studied.

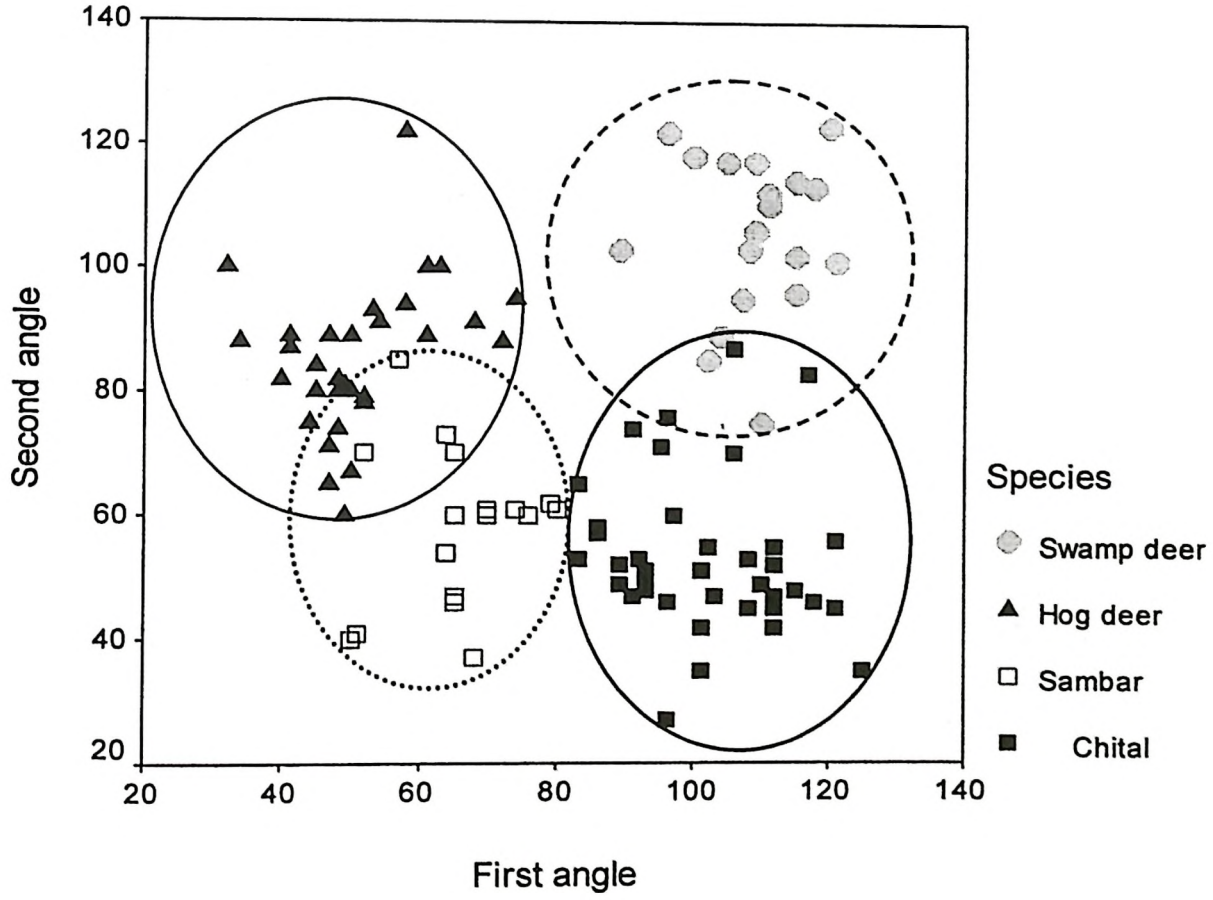


Figure 4.11. Scatter-plot of angles of antler of different species.

The angle measurements of first versus second angles were plotted, few overlaps were noticed (Fig. 4.11). Four clusters of chital, sambar, hog deer and swamp deer were found with few overlaps.

4.5.1.2. Statistical analysis

Quantitative measurements of antlers of four species namely chital, sambar, hog deer and swamp deer which did not had any missing values were used.

Discriminant Function Analysis

Discriminant function analysis was developed in order to distinguish antlers of different species. Discriminant function analysis was performed using stepwise method in which each of the variables was entered in a row and assessed for its significance of classification function using F statistic (i.e., F to remove 3.14). Since several of the variables would show multi-collinearity, Wilk's lambda values were used to ascertain the degree of collinearity and remove those variables from further analyses (Table 4.4).

Table 4.4.Variables used in the analysis.

Step	Variables	Tolerance	F to Remove	Wilks' Lambda
10	CICUMBEL	0.73	8.19	0.028
	SECTBL	0.63	36.72	0.046
	MAINL	0.42	12.66	0.031
	PEDICL	0.77	7.69	0.028
	CIRC5CMA	0.46	5.36	0.027
	FSTTINET	0.58	5.14	0.027
	ANGONE	0.57	9.07	0.029
	ANGTWO	0.43	6.07	0.027
	MAINB	0.41	5.20	0.027
	MAINT	0.54	4.49	0.026

Three functions could differentiate the antlers of all five species absolutely. First function itself could explain 57.3% variability (Table 4.5). Ten variables were used to derive three functions. The variables used in these three functions were as follows: Circumference below cornet (CICUMBEL), circumference at 5 cm from tip of second tine (SECTBL), length of main beam (MAINL), length of pedicle (PEDICL), circumference 5

cm above base (CIRC5CMA), circumference at 5 cm from tip of first tine (FSTTINET), first angle (ANGONE), second angle (ANGTWO), circumference at 5 cm from first branching on the main beam (MAINB) and circumference at 5 cm from tip of main beam (MAINT).

Table 4.5. Eigen values.

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	4.74	57.3	57.3	.91
2	2.25	27.2	84.4	.83
3	1.29	15.6	100.0	.75

Standardized Canonical Discriminant Function Coefficients

The discriminant functions for antlers of all the species are given in Table 4.6. The coefficients of these ten variables using Fisher's linear Discriminant functions were used to differentiate interspecific antlers (Table 4.6). The classification formula is: $D_i = C_i + (V_1 \times U_1) + (V_2 \times U_2) + (V_3 \times U_3) + \ldots\ldots + (V_n \times U_n)$.

where D_i = Discriminate score, C_i =Constant, V_1 = Value of the 1^{st} variable measured of the i^{th} sample and U_1 = Fisher linear coefficient value of the i^{th} sample for the i^{th} species. The unknown sample of antler was assigned to the species for which the discriminant score is the highest.

Table 4.6. Standardized Canonical Discriminant Function Coefficients.

Variable	Function	Function	Function
	1	2	3
CICUMBEL	.52	.14	.02
CIRC5CMA	.45	-.38	.05
PEDICL	.37	-.06	-.42
MAINB	.42	.46	.01
MAINL	-.31	-.68	.59
MAINT	-.18	.49	-.09
FSTTINET	.08	-.51	-.20
SECTBL	.05	1.06	-.059
ANGONE	.13	.41	.60
ANGTWO	-.11	-.50	-.49

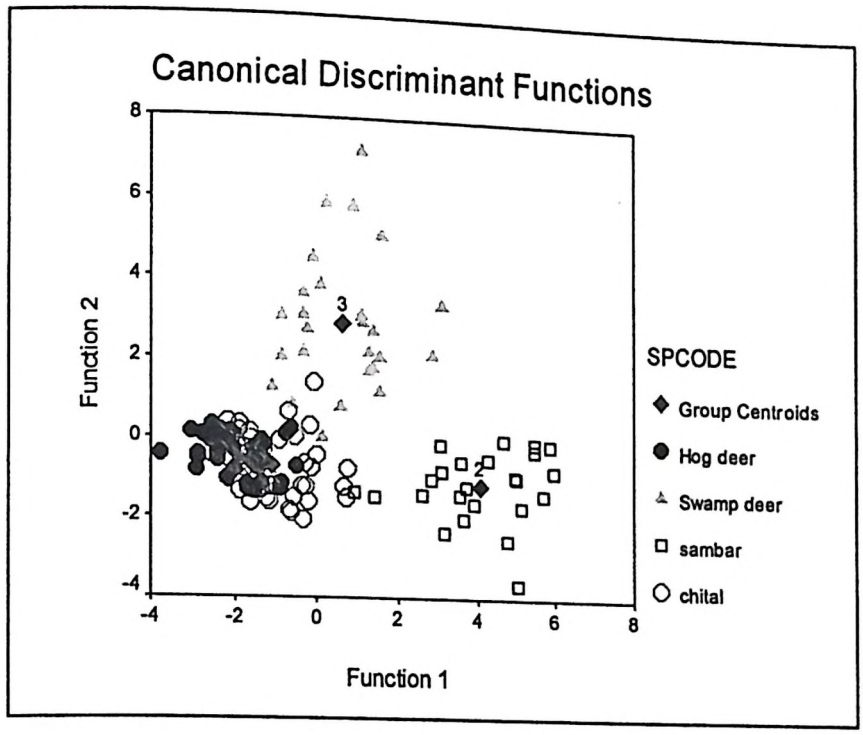


Figure 4.12 Canonical Discriminant Functions to distinguish antlers of various deer species.

Table 4.7. Fisher's linear Discriminant functions (Classification Function Coefficients).

Variables	Antlers			
	Chital (n=46)	Sambar (n=17)	Swamp deer (n=20)	Hog deer (n=31)
CICUMBEL	2.053	3.439	2.790	1.816
CIRC5CMA	.341	1.029	.159	.166
PEDICL	1.653	3.314	2.206	2.203
MAINB	.430	1.528	1.784	.272
MAINL	.291	.122	1.865E-02	.175
MAINT	-4.252E-02	-.504	.708	.219
FSTTINET	.150	.767	-.775	.393
SECTBL	-.460	-.484	.332	-.386
ANGONE	9.283E-02	7.453E-02	.127	3.220E-02
ANGTWO	.105	.124	5.413E-02	.156
(Constant)	-35.907	-81.989	-50.032	-29.201

Canonical Discriminant function was used to plot discriminant function 1 and 2 (Fig. 4.12). The group centroids of antlers formed four clusters however large overlap was noticed between chital and hog deer antler.

4.5.1.3. Cross-sections

Cross-sections of various antlers were noted. The cross-section of sambar antler had brownish yellow and granular core, chital had white core, where as swamp

deer antler had blackish core in center but, was not found consistently. Cross-section of hog deer and barking deer were not differentiated into core and cortex. Cross-section of hog deer was light yellow in colour, where as barking deer cross-section was exceptionally small.

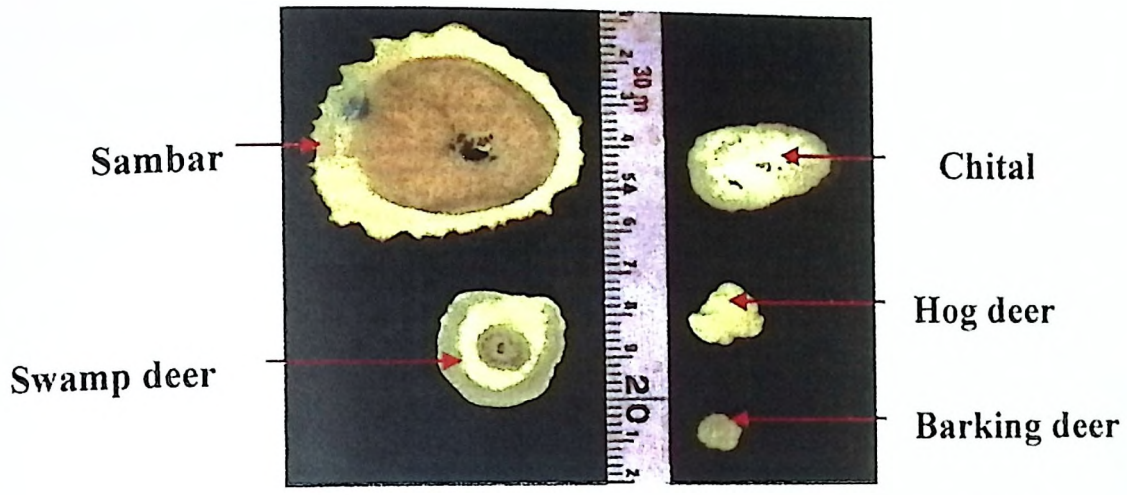


Figure 4.13. Cross section of antlers of different species.

4.5.2. Scanning electron microscopic characteristic

The surface topography of all antlers at outer portion was smooth except swamp deer which had smooth surface topography with few white lines (Fig. 4.14).

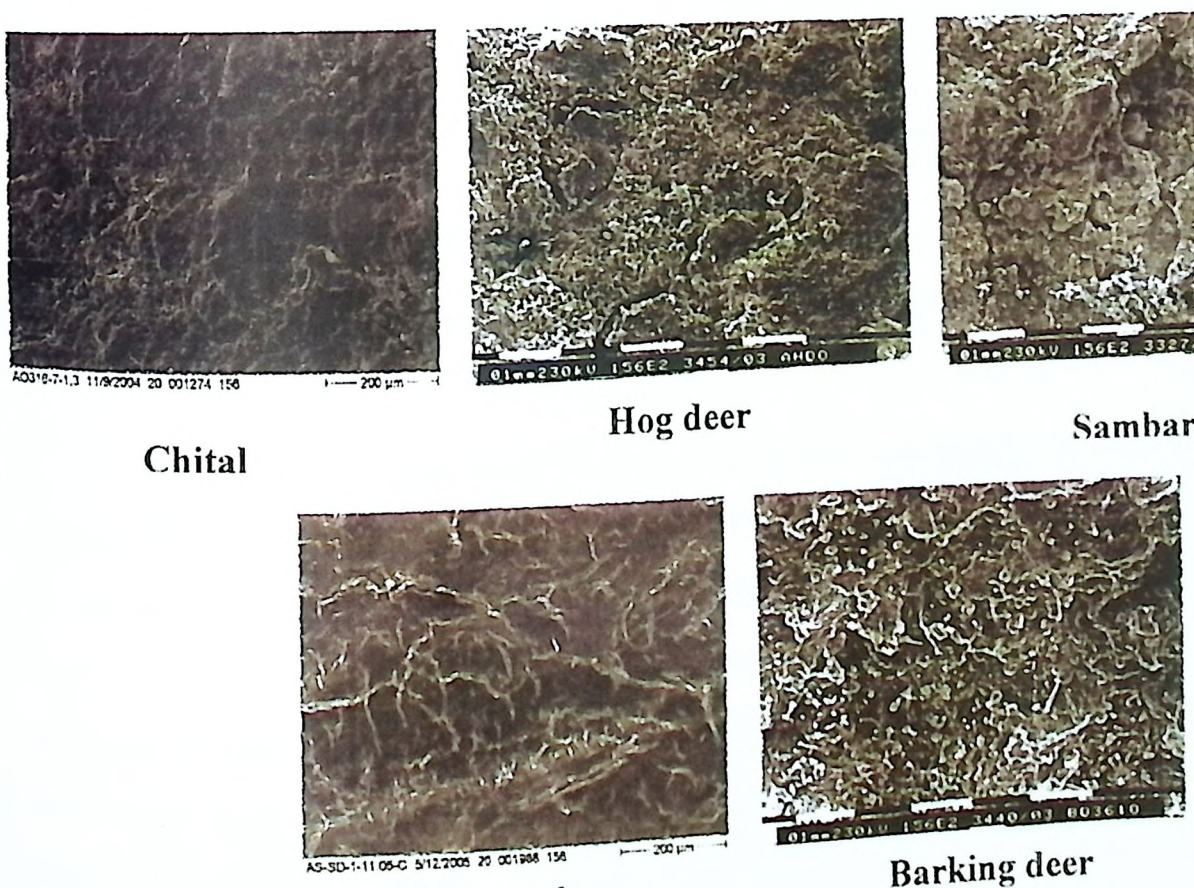
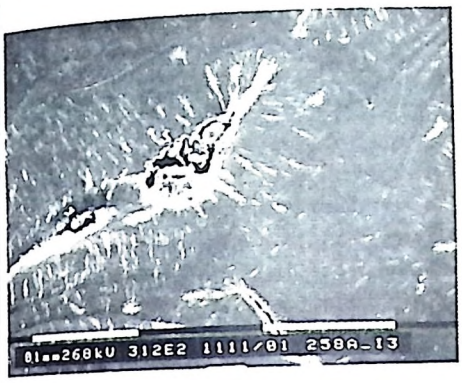


Figure 4.14. SEM micrographs of outer portion of antlers of selected deer species.

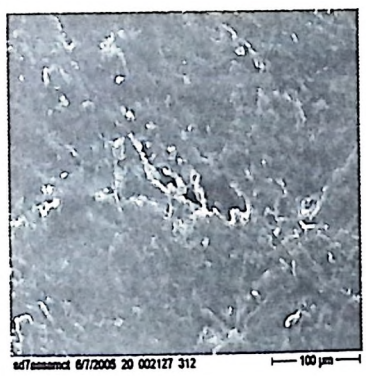
The surface topography of most of the antlers was different in cortex region. The micrograph of chital had smooth surface with few cracks and vesicles in between (Fig. 4.15). Sambar and hog deer had similar type of surface topography for cortex portion and was indistinguishable (Fig. 4.15). Surface of swamp deer was also smooth but few holes were visible (Fig. 4.15). Barking deer surface topography was distinct in having slanting lineages (Fig. 4.15).



Chital

Sambar

Hog deer



Swamp deer

Barking deer

Figure 4.15. Scanning micrographs at cortex portion of antlers of deer species.

Distinct differences were noted in the surface topography of core portion of antlers (Fig. 4.16). The surface topography of chital indicates that it smooth and star or variable shaped holes were present and these were surrounded by small whitish lines. In core of sambar, wave like pattern were visible. Fine circular lineations were observed at the core portion of hog deer. Swamp deer had circular holes on the surface of core portion. Barking deer had distinct with closely placed diagonal lineation like cortex portion.

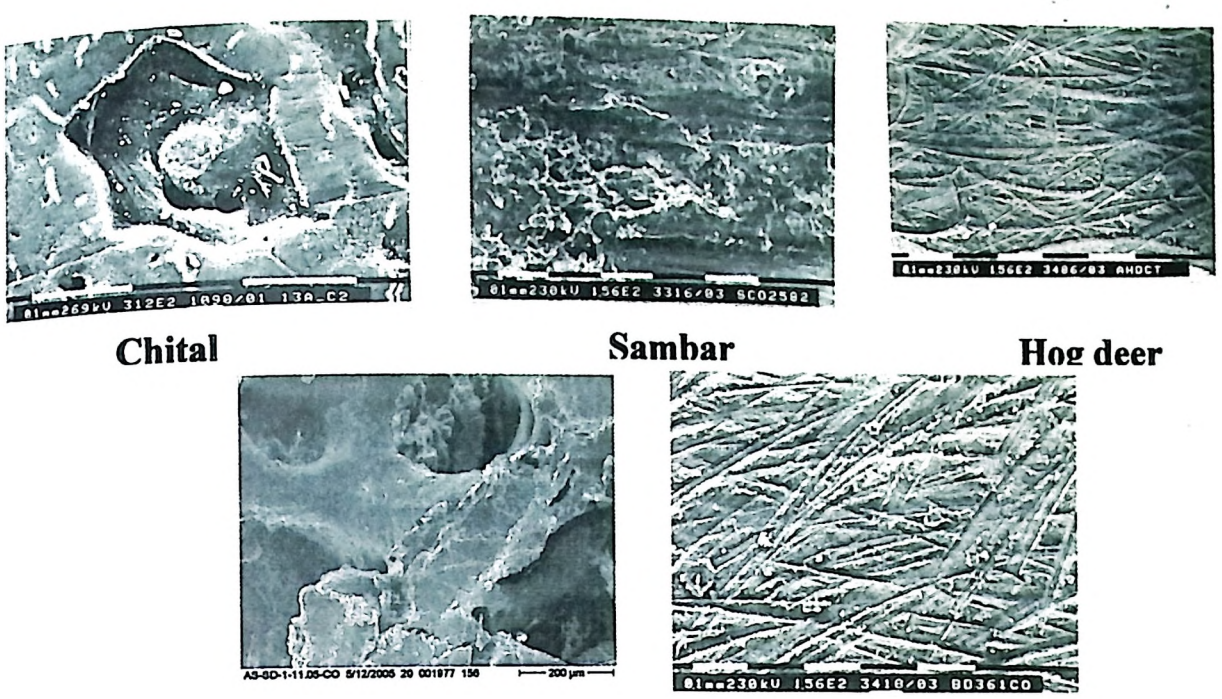


Figure 4.16. Scan micrographs patterns of antler at core portion of different deer species.

4.5.3. X-ray diffraction characteristics

Antler sample matched with hydroxyapatite minerals (American Standard Test Matching file no. 9, card number 432). Since ivory is also hydroxyapatite it was compared with antlers and a minute but distinct difference was noticed. Diffractogram of antler showed hump after 50^0 whereas in ivory a slanting line was observed at this position. Crystallite size of ivory ranged between ~ 16.9 – 86.1 nm where as in antler it ranged between ~ 26.5 –169.8 nm. Cell parameter 'a', 'c' and cell volume in case of antler (11.70, 6.92 and 820.22) was higher than ivory (9.49, 6.87 and 535.80). Scatter plot between maximum and minimum background intensity formed separate clusters for antler and ivory (Fig.4.17).

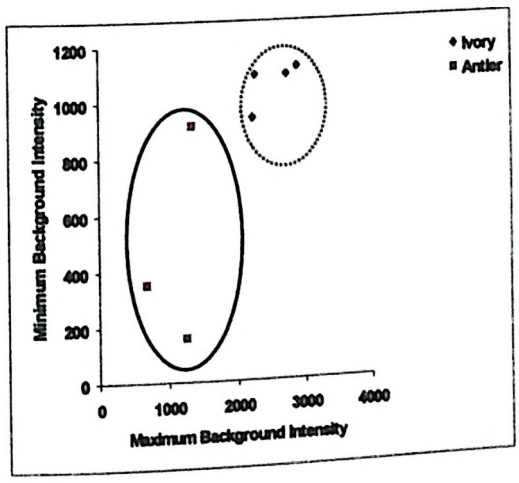
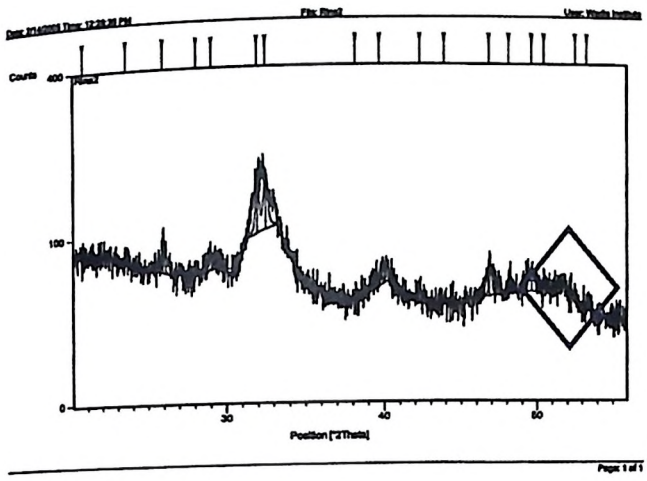
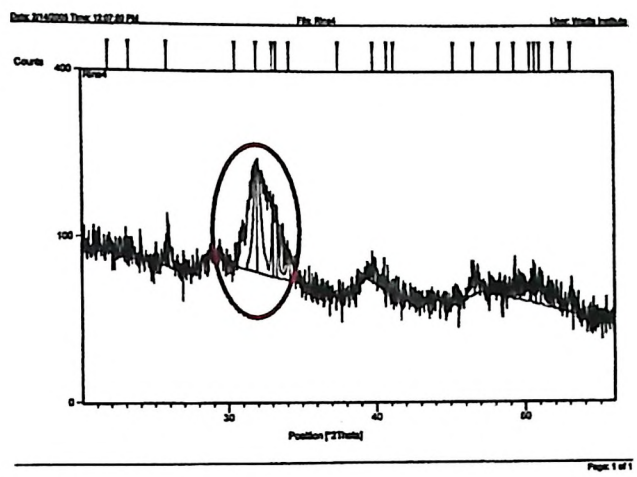


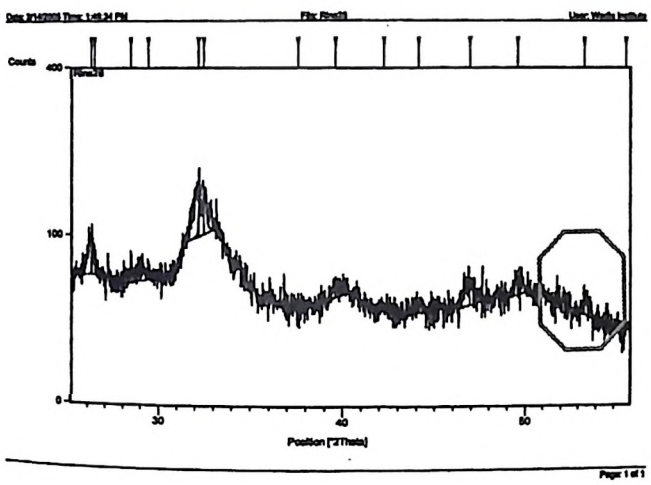
Figure 4.17. Observed difference among ivory and antler using background intensity.



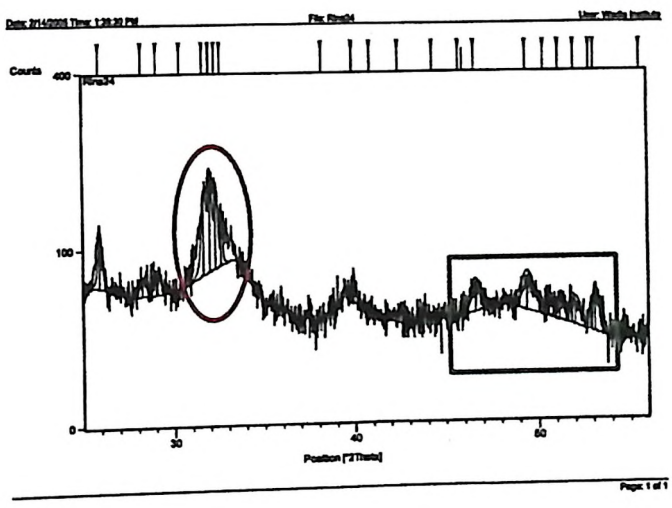
Chital



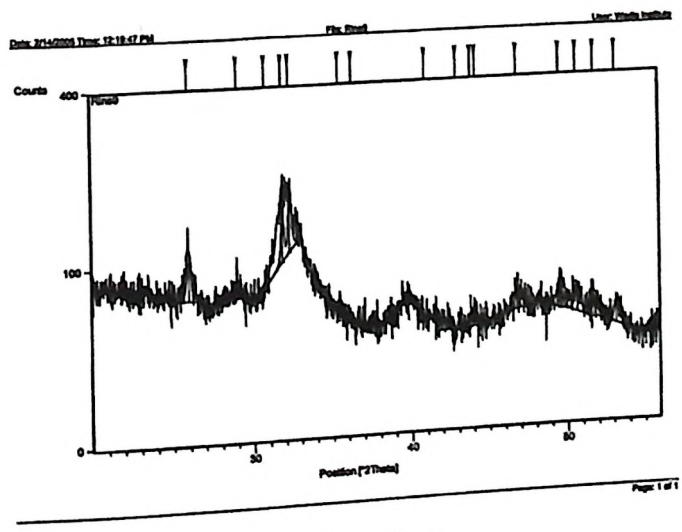
Sambar



Hog deer



Swamp deer



Barking deer

Figure 4.18. X-ray diffractogram of antlers of different deer species.

Broadly all deer species had similar XRD pattern matching with hydroxyapatite. There were minute differences in the diffractogram of these species but, these differences were however not consistent. In diffractogram of chital, very steep decline was noticed after 50^0 which were not observed in diffractogram of any other deer species. In swamp deer, the main peak at 31^0 had shoulder peaks in some samples and had well developed continuous peak after 46^0. Barking deer had small peak at 35^0 which was not present in any other species. In most of the samples of hog deer there were shoulder peak adjacent to 40^0. In sambar, no such distinct feature was noted except well developed peak at 31^0 (Fig.4.18).

All species of deer antlers examined had peak at 25^0, 32^0, 46^0, and 53^0. Swamp deer had maximum number of peaks. Other then common peaks, there were peaks at 26^0, 28^0, 32^0 (an extra peak in few samples of swamp deer were noted), 34^0, 39^0,40^0,44^0,47^0,49^0 and 50^0. Some of the peaks were only present in swamp deer like 34^0 and 44^0. Barking deer had peaks other than common peaks at 28^0,39^0 and 50^0. Barking deer only missed the peaks at 26^0, 49^0 (Table 4.8). Other than common peak hog deer had peaks at 26^0, 28^0, 31^0, 39^0 and 49^0. Chital had extra peaks other than common peaks at 26^0, 29^0, 31^0, 40^0, 47^0 and 49^0. Only chital did not have any peak at 39^0. Sambar had extra peaks at 26^0, 29^0, 31^0, 39^0, 40^0, 42^0 and 49^0 (Table 4.8). Based on presence and absence of peaks at different angles dendrogram was generated to differentiate antlers of deer species based on cluster analysis (Fig. 4.19).

Table 4.8. Mean and range of relative intensity of peaks at various angles of five deer species.

Angle	Chital		Sambar		Swamp deer		Hog deer		Barking deer	
	Mean Relative intensity	Range	Mean Relative intensity	Range	Mean Relative intensity	Range	Mean Relative intensity	Range	Mean Relative intensity	Range
25	47.91	0-35.14	41.75	0-69.27	52.73	0-55.41	45.13	0-55.80	49.50	49.50
26	55.08	0-66.97	58.83	0-86.23	56.43	0-60.62	40.29	0-43.62		
28					7.30	0-7.30	13.34	0-15.15	13.80	13.80
29	12.10	0-13.79	23.88	0-33.07						
31	100	0-100	100	0-100			100	0-100		
32	100	0-100	100	0-100	96.15; 100	0-98.79;100	100	0-100	100	100
34					14.92	0-15.80				
39			16.66	0-21.32	12.42	0-13.60	16.32	0-18.61	9.81	9.81
40	16.84	0-17.59	16.56	0-16.56	10.03	0-10.03				
42			12.36	0-12.36						
44					2.79	0-2.80				
46	11.60	0-15.82	15.48	7.11-31.32	8.5	0-9.06	11.23	0-13.06	9.02	9.02
47	13.85	0-13.85			10.33	0-10.33				
49	9.34	0-10.93	23	0-34.58	9.54	0-10.97	9.49	7.88-10.78		
50			9.66	0-12.98					9.41	9.41
53	7.30	0-7.30	15.39	0-25.81	8.17	0-9.05	8.68	0-9.46	7.92	7.92

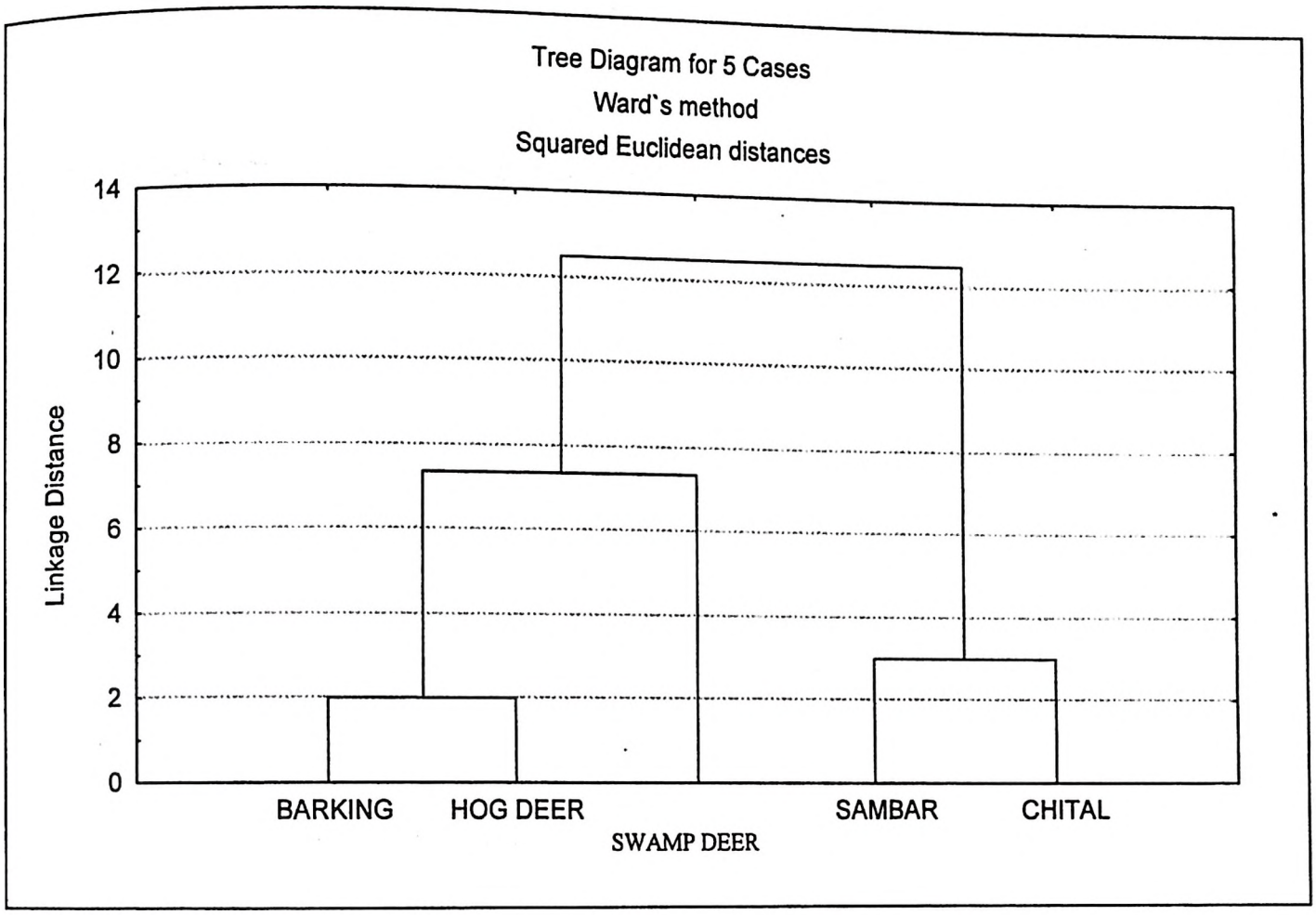


Figure 4.19 Dendrogram generated using cluster analysis indicating differences among deer species based on presence and absence of x-ray diffraction peaks in antler.

4.5.4. Thermo gravimetric characteristics

Fig. 4.20 indicates the nature of weight loss in antlers of different species with constant increase in temperature. Maximum weight loss was noted between 200 and 400^0C in antlers of all species. Weight loss pattern of antlers of different species showed remarkable differences in three temperature sections, 0-300^0C, 450-700^0C and 1000-1400^0C. In section 0-300^0C, the weight loss was least in sambar (985.1ug), followed by chital (1395.93 ug), swamp deer (1598.35 ug), hog deer (1656.41 ug) and highest in barking deer (1711.61 ug). In temperature section 450-700^0C, the weight loss in sambar (2394.8 ug) was lowest followed by swamp deer (4015.9 ug), chital (4188.4 ug), hog deer (4356.1 ug) and barking deer (4491.02 ug). In the temperature section 1000-1400^0C, the weight loss in sambar (3079.98 ug) was again least followed by hog deer (4616.83 ug), barking deer (4733.64 ug), chital (5013.54 ug) and swamp deer (5226.16 ug).

Thermo gravimetric data analysis of antlers belonging to different species indicated variation in percentage water loss at 100^0C and the percentage water loss pattern in ascending order was chital > barking deer > sambar > hog deer > swamp deer. Total percentage weight left till 1400 ^{0}C was highest in antlers of hog deer followed by chital, sambar, swamp deer and barking deer (Fig.4.21).

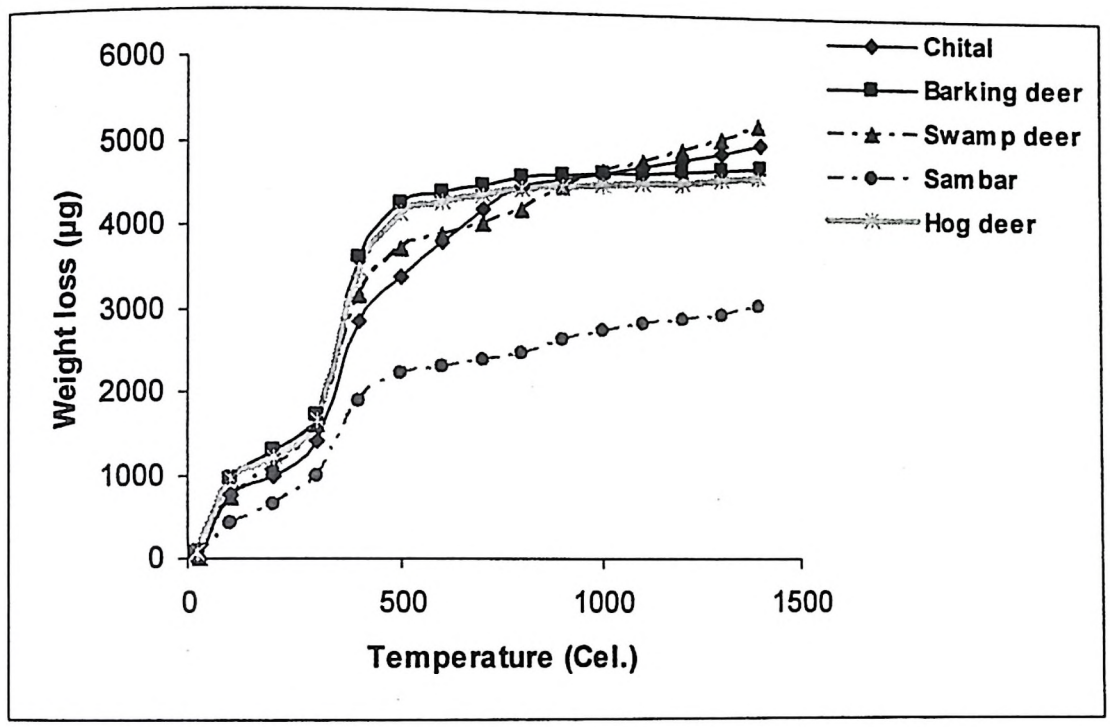


Figure 4.20. Nature of weight loss in antlers of deer species.

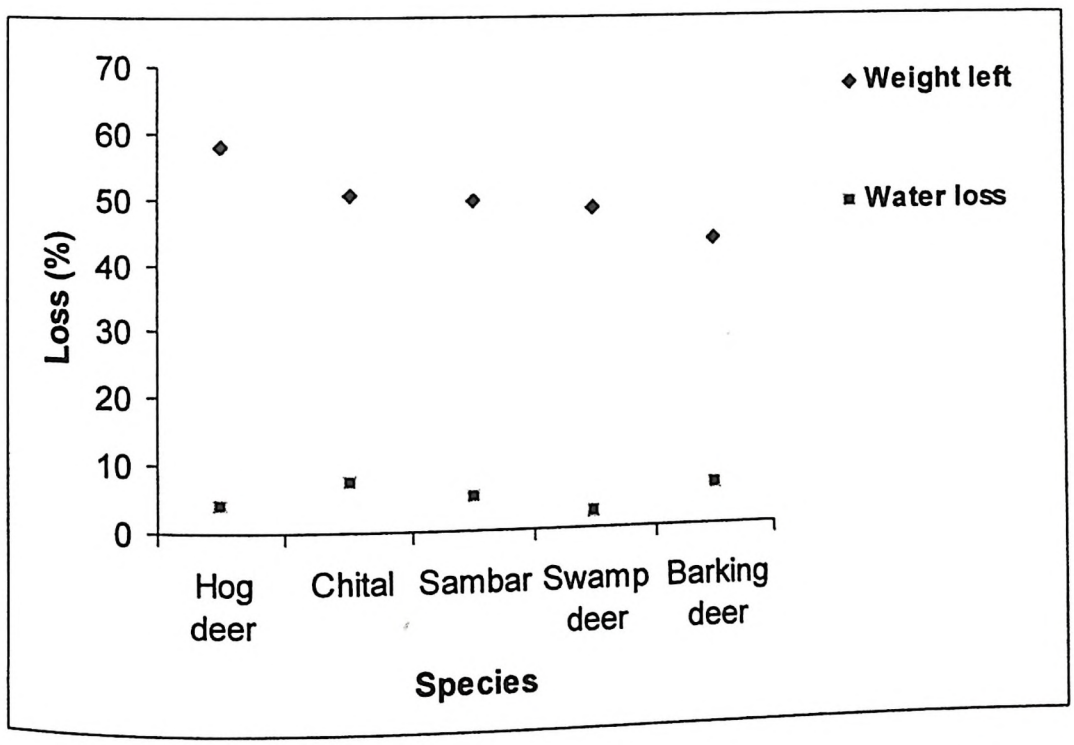
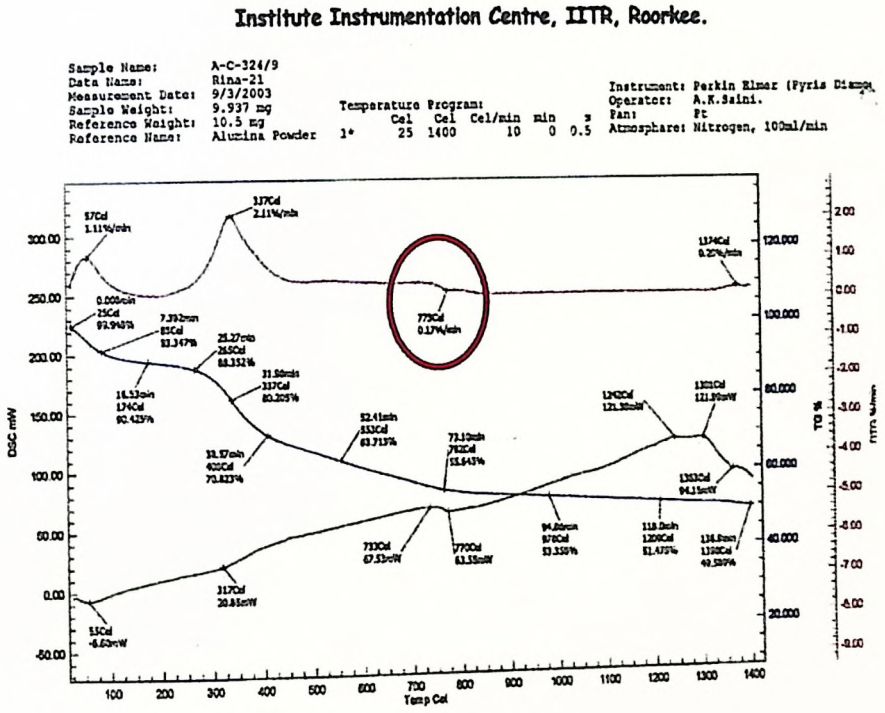
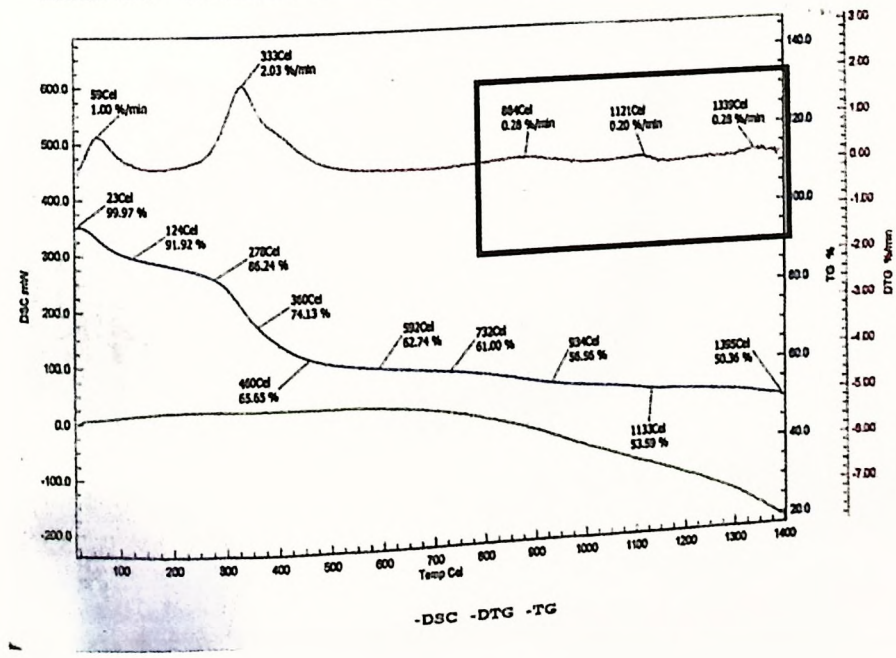


Figure 4.21. Percent weight left and water loss from antler of different deer species.

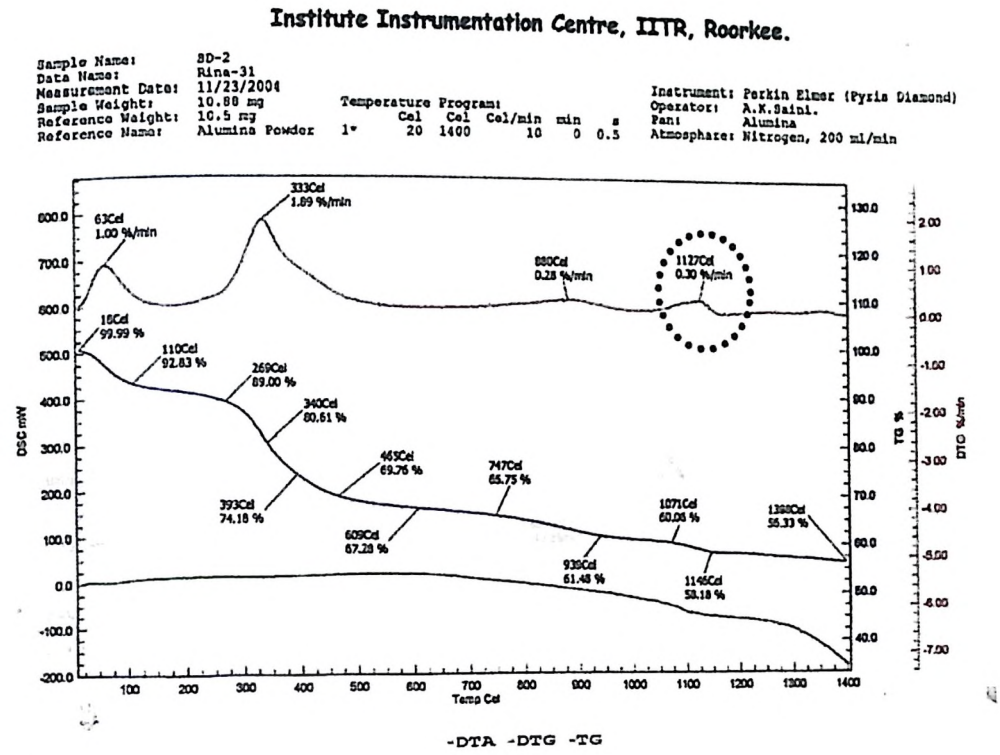
Thermographs of antlers of various species given in Fig. 4.22 indicate minute but consistent differences. Endothermic peak was noticed at 770-810 ^{0}C only in chital, where as in sambar there were three consecutive exothermic peaks at 884 ^{0}C, 1120 ^{0}C and 1339 ^{0}C. In antler of swamp deer, two peaks were noticed between 800 and 1135 ^{0}C. There was slight difference in thermographs of barking deer and hog deer, only a minute exothermic peak was noticed in barking deer at 150 ^{0}C, where as in hog deer peak was absent.



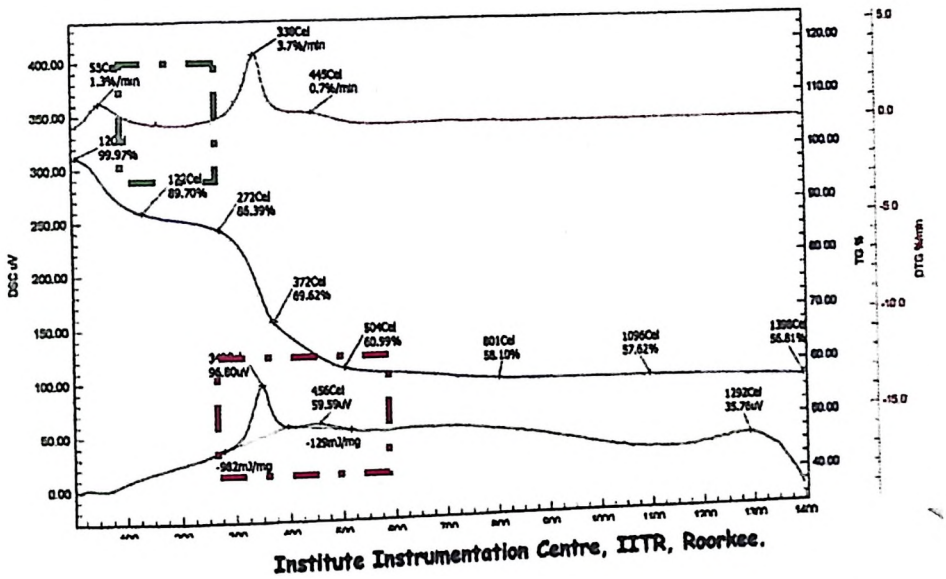
Chital



Sambar



Swamp deer



Barking deer

Hog deer

Figure 4. 22. Thermographs of antlers of different deer species.

4.5.5. Elemental analysis

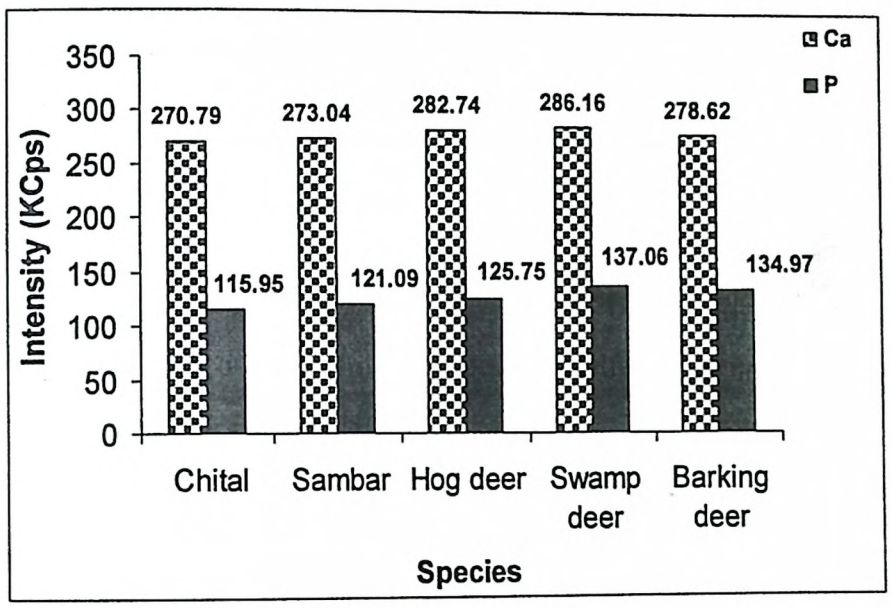
4.5.5.1. X-ray fluorescence

The qualitative elemental fluorescence X-ray screening from antlers of five species reveals that the intensity (KCps) of calcium (Ca) was highest in all species out of the seventeen elements analyzed. Major intensities were of calcium (Ca), phosphorous (P), strontium (Sr), iron (Fe), silicon (Si), sodium (Na) and magnesium (Mg). Minor intensities were of sulphur (S), zinc (Zn), chlorine (Cl), copper (Cu), aluminum (Al) and potassium. Intensities of chromium (Cr), nickel (Ni), manganese (Mn), and rare earth elements gadolinium (Gd) and osmium (OS) were in traces (Fig. 4.23). Significant difference in intensities of calcium, phosphorous, zinc, chlorine, iron, sodium, silicon and strontium content were noticed and there were slight difference in copper, potassium, sulphur, aluminum, magnesium and silicon contents.

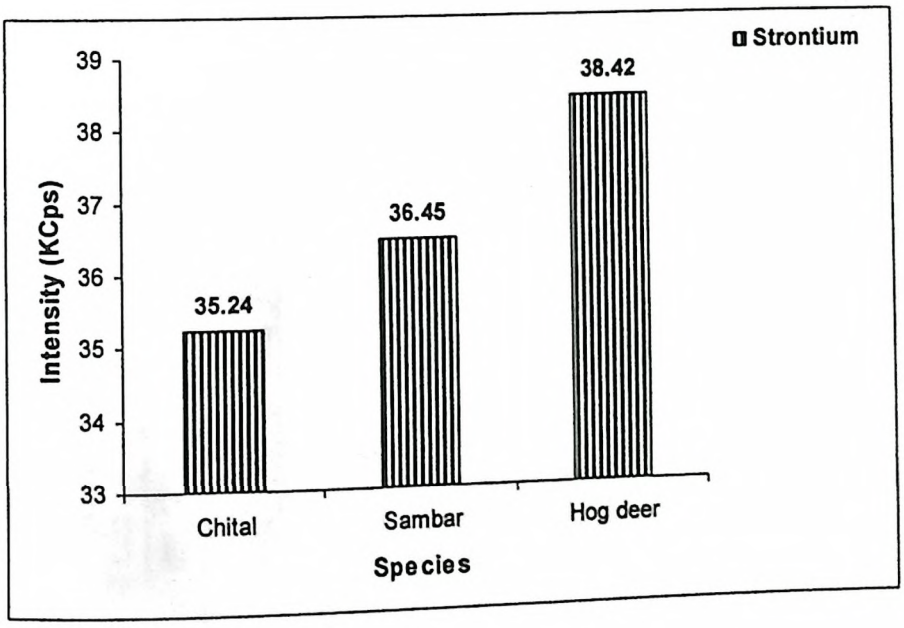
XRF intensity (KCps) of calcium was highest in swamp deer followed by hog deer, barking deer, sambar and chital. Intensity of phosphorous was highest in swamp deer followed by barking deer, hog deer, sambar and chital (Fig.4.23 a). Intensity of strontium was highest in antler of hog deer followed by sambar and chital, however this was not detected in remaining two species (Fig.4.23 b). Silicon intensity was highest in antler of sambar followed by barking deer, swamp deer, chital and hog deer. Intensities of iron were highest in swamp deer followed by barking deer, chital hog deer and sambar. Sodium intensity was highest in hog deer, sambar, chital and quite low in barking deer and swamp deer. Magnesium intensities were almost equal in antlers of all deer species (Fig. 4.23 c). Intensity of sulphur was highest in antler of chital followed by sambar, swamp deer, barking deer and in hog deer, only slight differences were observed. XRF intensity of chlorine is highest in antler of chital followed by sambar, barking deer, hog deer and quite low in swamp deer. Intensity of aluminum was higher in antlers of sambar and hog deer compared to antlers of other three species, where as the differences were negligible. Intensity of zinc was highest in antler of sambar followed by chital and barking deer and almost equal in hog and swamp deer. The intensity of copper in antlers of different species had little variation. The intensity of potassium was distinctly high almost more than double in antler of barking deer and sambar as compared to chital and hog deer, and swamp deer antler had lowest intensity of potassium (Fig. 4.23d). Intensities of chromium, manganese,

nickel, osmium and gadolinium however could not be detected in chital. Intensity of chromium was highest in swamp deer followed by barking deer and sambar, however it was absent in antlers of two species viz. chital and hog deer. The intensity of manganese was detected four times higher in sambar compared to antlers of hog, swamp and barking deer, these had almost equal intensity. Osmium intensity was highest in barking deer followed by sambar, hog deer and swamp deer but, the differences were slight. Intensity of gadolinium was only detectable in sambar and hog deer (Fig. 4.23 e).

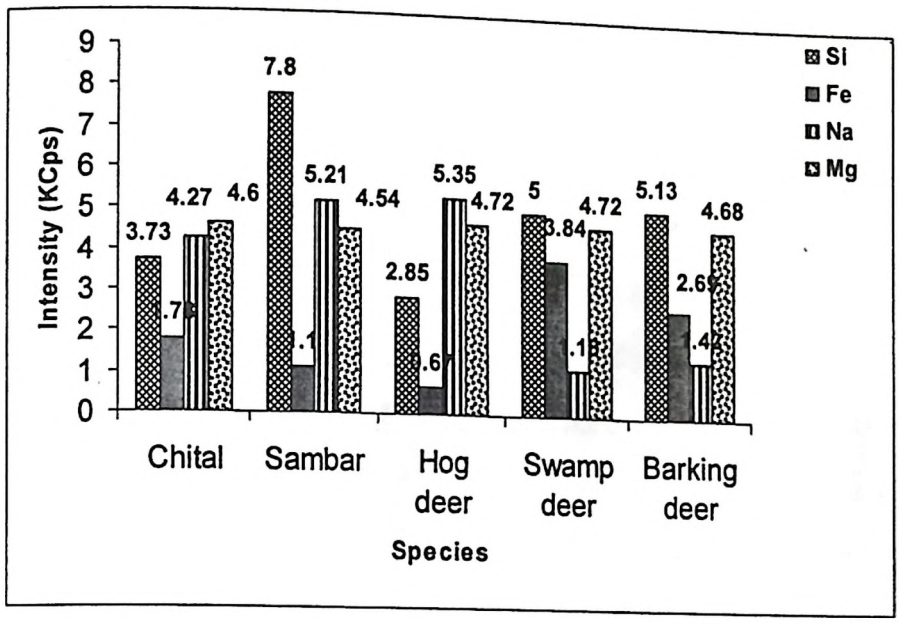
a



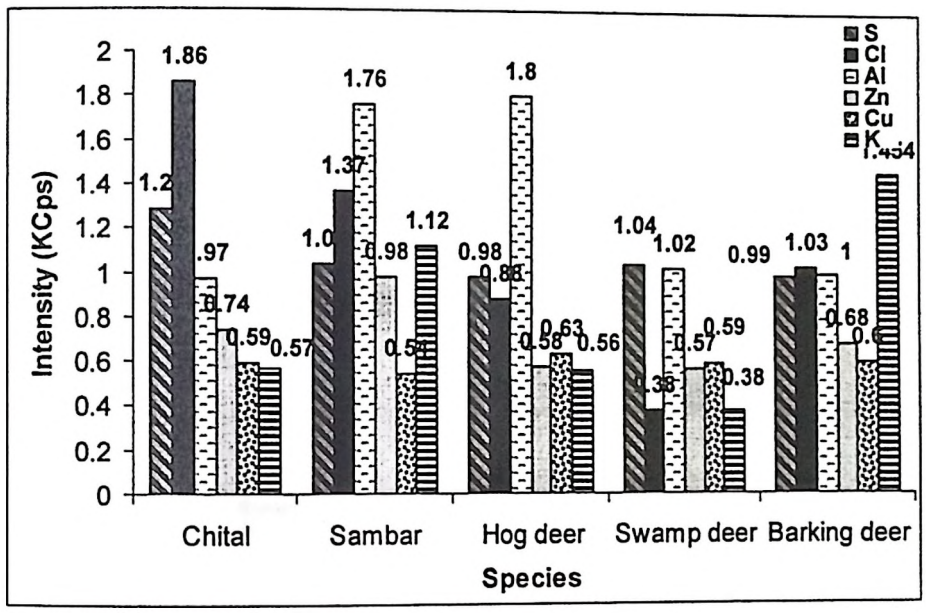
b



c



d



e

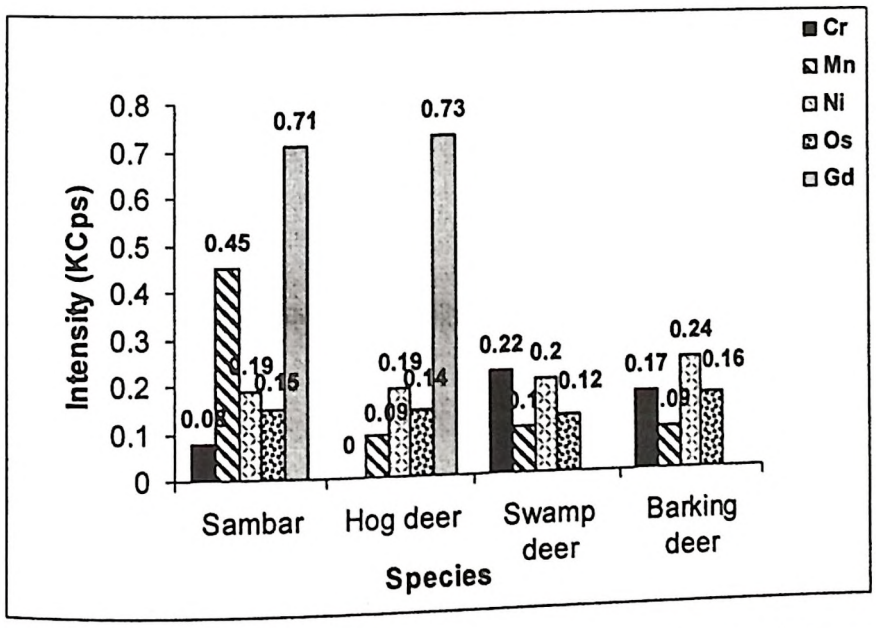


Figure 4.23. XRF intensity (KCps) of elements in antler of different deer species.

4.5.5.2. Inductively coupled plasma – mass spectrometry

Uranium and cadmium was not detected in antler samples of any species. Molybdenum was detected only in few antlers. Table 4.9 indicates variation were present in mean concentration in antlers of different deer species analyzed but, the range of concentrations of most elements analyzed were found to be overlapping in antlers of deer species, only few differences were found. The amount of potassium and strontium was remarkably high in barking deer antler. The concentration of barium was on higher side in swamp deer only with a slight overlap in sambar, while barium is less variable in hog deer. The concentration of iron and nickel were higher in sambar antler as compared to other deer species. Concentration of copper was lower in antler of chital compared to other antlers.

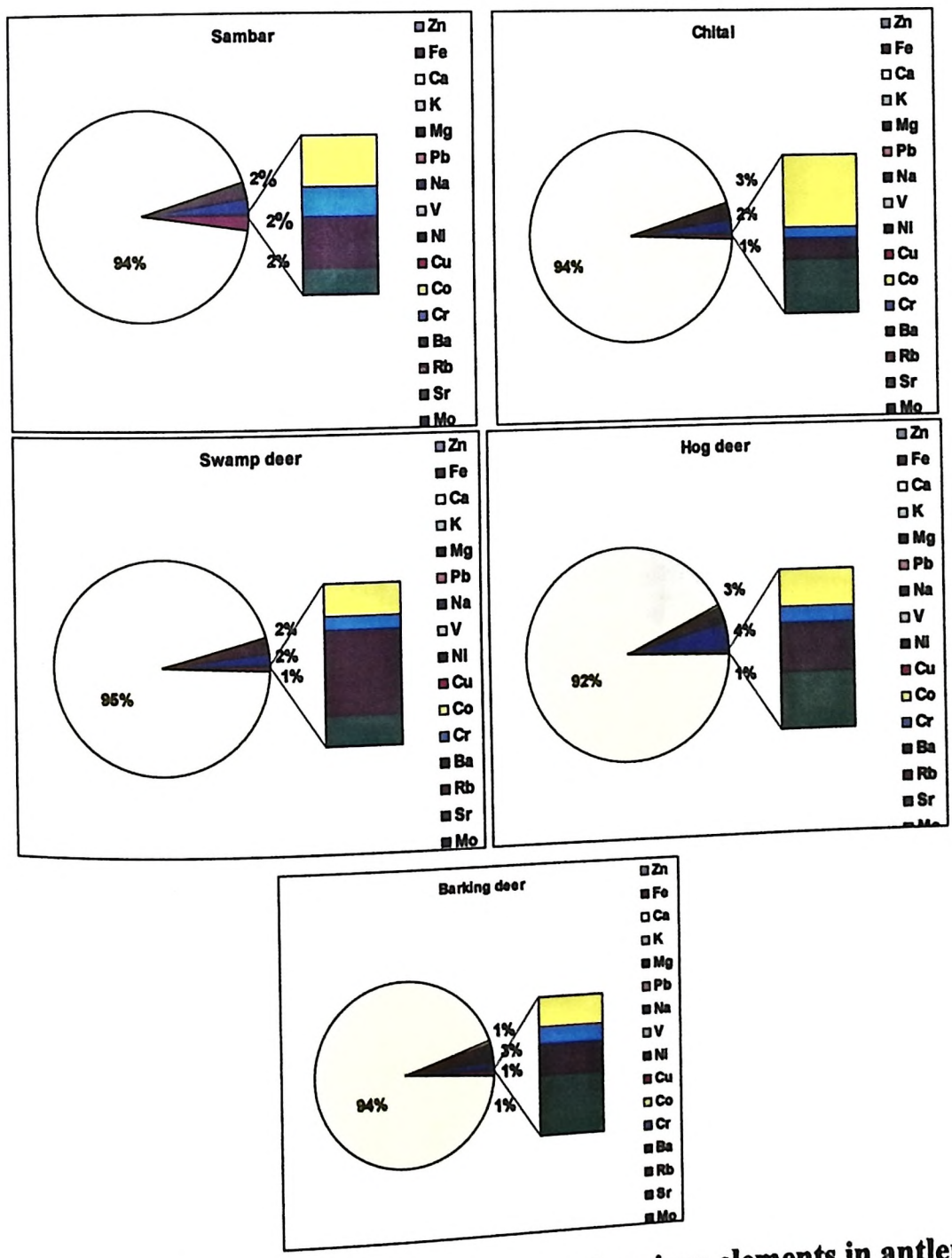


Figure 4.24. Composition of mean concentrations of various elements in antlers of deer species.

***Table 4.9. Concentrations of various elements* (mean±SD) in ppm detected in antlers of different deer species by ICP-MS.**

Elements	Chital (n=7)		Sambar (n=4)		Swamp deer (n=4)		Hog deer (n=4)		Barking deer (n=1)
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Zn	57.58± 17.29	30.63 – 76.16	80.48± 33.92	61.51- 131.27	43.27± 25.73	13.69 – 70.24	44.3± 4.65	40.04 - 50.70	102.9
Fe	1397.93± 901.52	779.53 – 3317.01	4077.17± 5680.63	876.86 - 12574.31	1076.96± 175.25	939.4 – 1329.38	861.73± 72.65	779.29 - 950.71	1688.31
Ca	175794.19± 23827.7	139367.39 – 208621.3	166286.56± 23992.67	141104.11 - 192042.6	151760.89± 51765.27	74499.53 - 183293.70	144662.31± 48018.11	73065.92 - 175518.98	174376.45
K	308.68± 236.26	172.11 - 840.39	386.34± 201.07	196.3 - 669.88	145.58± 40.76	99.71 – 184.39	344.27± 155.48	113.32 - 442.66	1340.66
Mg	4820.11± 707.9	3909.99 - 5804.92	4139.14± 628.9	3604.24 - 5039.3	3922.53± 1180.52	2195.85 - 4857.56	4144.88± 1262.63	2274.36 - 5044.76	5729.26
Pb	10.29±7.62	0 –19.88	17.68±6.12	10.55 – 24.07	6.88±7.68	0.98 - 15.83	16.82±26.66	1.89 - 56.66	17.98
Na	4032.11± 902.19	2964.70 - 5479.24	4154.36± 571.38	3494.04 - 4888.99	2741.67± 744.47	1686.45 - 3396.88	6180.27± 4434.03	1371.78 - 9996.24	2208.79
V	51.76±29.19	8.89 - 82.68	68.81±11.88	54.56 – 83.01	34.86±38.61	0 – 70.24	14.86±25.25	0.95 –52.68	57.94
Ni	4.08±2.97	0.99 - 8.81	9.93±10.3	1.92 - 24.13	3.7±2.47	0.98 - 6.92	2.7±2.37	0.95 - 5.96	6.99
Cu	9.01±5.08	0.99 - 13.92	13.44±7.85	8.63 - 25.1	15.05±10.7	0 - 24.73	6.89±7.77	0.95 - 17.89	14.99
Co	122.72±71.54	28.66 - 200.79	131.4±24.52	95.24 - 149.57	82.77±83.18	6.95 - 157.27	44.75±70.96	5.71 - 151.09	149.85
Cr	17.61±11.3	8.86 - 39.41	77.89±138.56	7.94 -285.71	30.51±6.86	25.82 - 40.55	20.64±9.75	10.93 - 30.33	88.91
Ba	33.03±22.03	13.85 - 75.49	128.57±74.79	47.94 - 225.2	207.55±58.27	150.54 - 288.82	55.42±4.53	51.76 - 61.63	143.86
Rb	0.42±0.53	0-0.98	0.97±0.79	0 - 1.98	0.49±0.99	0-1.98	0.95±1.1	0-1.90	5.99
Sr	90.18± 37.44	45.45 - 157.64	64.79±16.84	50.19 - 82.45	71.79±28.43	42.53 - 100.49	66.34±25.02	41.75 - 89.10	289.71
Mo	0.42± 0.78	0-1.98	0.48±0.97	0 - 1.93	0.25±0.49	0 - 0.99	0.25±0.5	0-0.99	0.10

The mean elemental concentrations of antlers of various species indicate that the major constituents are calcium (95% in antlers of swamp deer, 94% in antlers of chital, sambar and hog deer and 92% in barking deer) whereas, other major constituents were magnesium (3% in antlers of chital, hog deer and barking deer, 2% in sambar and swamp deer), sodium (4% in antlers of hog deer, 2% in chital, sambar and swamp deer and 1% in barking deer), iron (2% in antler of sambar and 1% in antlers of all other deer) and potassium (1% in barking deer). Other elements were below 1% level (Fig. 4.24). However, phosphorous could not be analyzed.

4.5.5.3. Statistical analysis

Quantitative concentrations of antlers of five species namely chital, sambar, hog deer, barking deer and swamp deer detected through ICP-MS were used.

Discriminant Function Analysis

Discriminant function analysis was developed in order to distinguish antlers of different species based on their elemental concentration. Since several of the variables would show multi-collinearity, Wilk's lambda values were used to ascertain the degree of collinearity and remove those variables from further analyses.

Four functions could differentiate the antlers of all five species absolutely. First function itself could explain 87.9% variability (Table 4.10). Fifteen variables were used to derive four functions. The variables (elements) used in these functions are given in Table 4.11.

Table 4.10. Eigen values.

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	225.230	87.9	87.9	.998
2	21.334	8.3	96.3	.977
3	6.133	2.4	98.7	.927
4	3.401	1.3	100.0	.879

Standardized Canonical Discriminant Function Coefficients

The discriminant functions of elemental concentrations of antlers of five species are given in Table 4.12. The coefficients of these variables using Fisher's linear Discriminant functions were used to differentiate interspecific variation in antlers of deer species (Table 4.12). The classification formula is: $D_i = C_i + (V_1 \times U_1) + (V_2 \times U_2) + (V_3 \times U_3) + \ldots + (V_n \times U_n)$.

where D_i = Discriminate score, C_i =Constant, V_1 = Value of the 1st variable measured of the i^{th} sample and U_1 = Fisher linear coefficient value of the i^{th} sample for the i^{th} species. The unknown sample of antler was assigned to the species for which the discriminant score was highest.

Table 4.11. Standardized Canonical Discriminant Function Coefficients.

Variable	Function 1	Function 2	Function 3	Function 4
Zn	4.855	-2.743	2.571	-.693
Fe	6.557	2.445	4.489	-2.471
Ca	-4.573	3.138	-2.528	3.261
K	10.128	1.499	.064	1.770
Mg	7.454	-.304	2.588	-1.070
Pb	13.420	1.414	1.811	.940
Na	4.860	.387	-.459	.354
V	1.433	.070	-2.583	1.025
Ni	-10.693	-3.120	-3.730	1.519
Cu	4.062	1.839	-.896	.470
Co	-9.599	2.001	.318	.533
Cr	-5.686	1.937	-1.865	.949
Ba	.416	1.986	1.565	.149
Sr	-10.794	-2.370	.128	-1.253
Mo	-7.167	-1.638	-.512	-.103

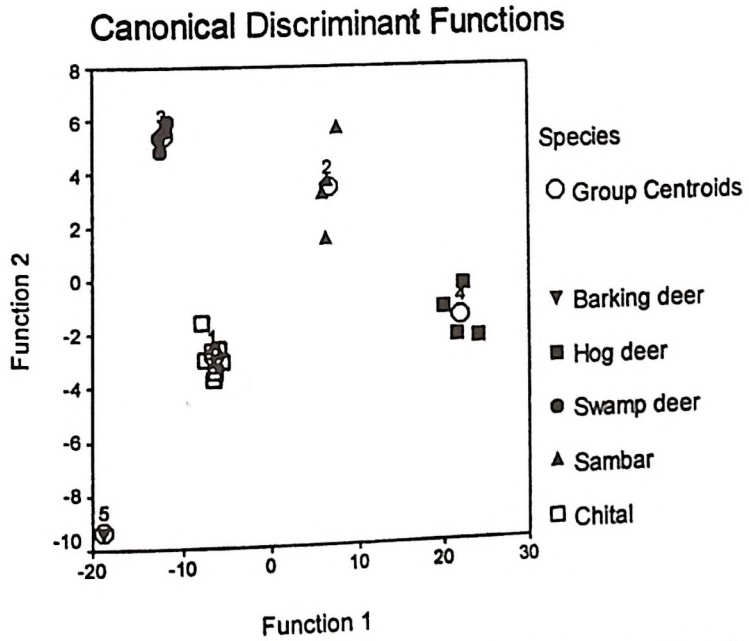


Figure 4.25 Canonical Discriminant Functions to distinguish antlers of different deer species.

Table 4.12. Fisher's linear Discriminant functions (Classification Function Coefficients).

Variables	Antlers				
	Chital	Sambar	Swamp deer	Hog deer	Barking deer
Zn	.420	2.711	-1.390	6.986	-.445
Fe	3.177E-02	7.206E-02	3.284E-02	.112	9.102E-03
Ca	3.069E-04	-6.989E-04	1.347E-03	-3.501E-03	7.981E-04
K	.874	1.662	.629	2.414	.188
Mg	.131	.237	9.541E-02	.370	5.964E-02
Pb	16.256	30.465	11.986	45.075	4.893
Na	3.903E-02	7.071E-02	2.673E-02	.105	8.033E-03
V	.318	.909	-.310	1.441	-1.090
Ni	-26.859	-58.565	-23.287	-89.310	-3.976
Cu	12.325	20.849	10.864	27.548	3.268
Co	-1.024	-2.683	1.717E-02	-5.061	.572
Cr	-.744	-1.771	-.106	-3.425	-8.402E-02
Ba	.839	1.331	1.272	1.273	.763
Sr	-5.946	-11.302	-4.528	-16.288	-1.097
Mo	-146.309	-293.195	-112.311	-434.983	-18.027
(Constant)	-330.718	-711.522	-390.126	-1112.824	-310.842

Canonical Discriminant function was used to plot discriminant function 1 and discriminant function 2 (Fig. 4.25). The group centroids of antlers formed five distinct clusters.

4.5.6. DNA Techniques

The DNA yield from the three extraction methods were as follows (1) Phenol Chloroform Isoamyl method yielded highly degraded and less than 100bp DNA. This quality of DNA was not sufficient for Polymerase Chain Reaction (PCR). (2) Commercially available Gene Clean Kit for Ancient Bone DNA (Bio 101® Systems, Q-BIOgene, USA), yielded 200bp to 300bp DNA. The obtained DNA was degraded, so gel purification method (Qia Quick® Gel Extraction Kit, QIAGEN Gmbh) was used to purify the DNA product. This purification yielded a better DNA product but less than 150bp. In view of available primers for mitochondrial cytochrome 'b' gene, this DNA product size was not suitable for PCR amplification and further analysis. (3) Sodium Acetate precipitation method yielded a better quality of DNA (150bp-180bp)

from different target region of antlers. DNA extraction was successful from outer portion and core portion (Fig. 4.26).

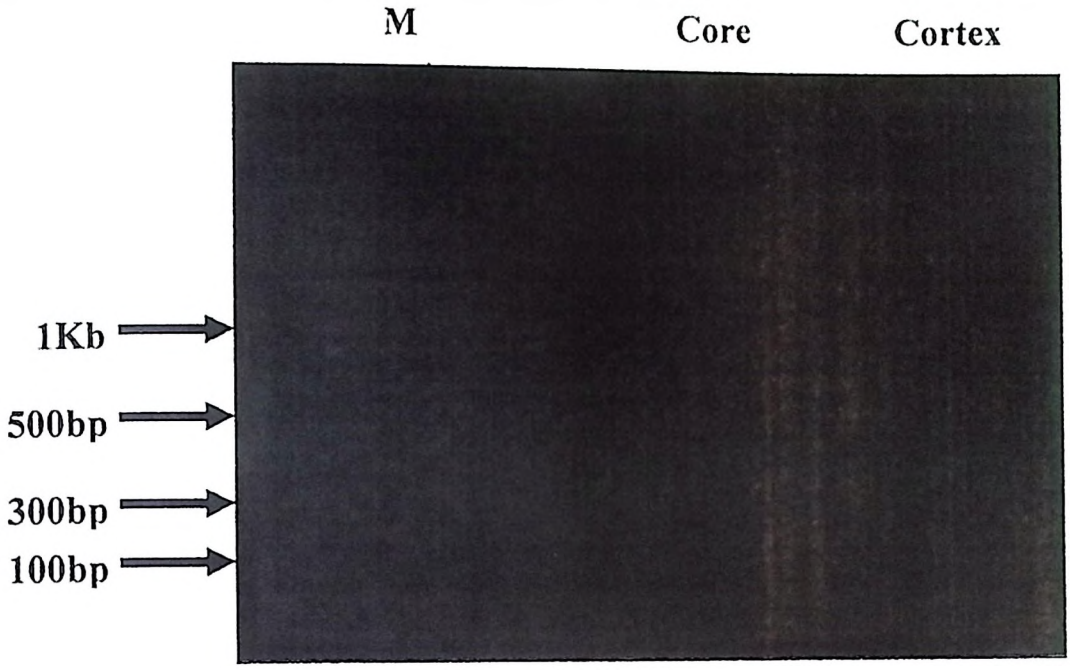


Figure 4.26. DNA extraction from antlers using sodium acetate precipitation method (M- Molecular marker of 1Kb ladder).

In view of these results, there is a strong need to standardize this method/protocol for cortex and basal portions for better yield. Failure of PCR amplification showed that there might be PCR inhibitors. So DNA product was purified by using gel purification method (Qia Quick® Gel Extraction Kit, QIAGEN Gmbh) and direct column purification method (Qia Quick® PCR Purification kit, QIAGEN Gmbh). Gel purification method showed better quality and quantity than the

direct column purification method. DNA (8 ul) was visualized under the ultra violet light. Best extraction was possible from sambar antler and worse was from chital antler.

Amplification with only cytochrome b gene 186bp (Farell *et al.* 2000) was possible as 381bp cytochrome b gene (Kocher 1989) could not amplify (Fig. 4.27). More standardization is required to extract DNA from antler and amplify. Above mentioned DNA extraction methods were applied with samples of target antler regions of sambar (Table 4.13). Since the DNA yield was better with sodium acetate precipitation method than the other methods. Therefore, this method was used to extract DNA from target antler regions (Fig. 4.26) and Table 4.14.

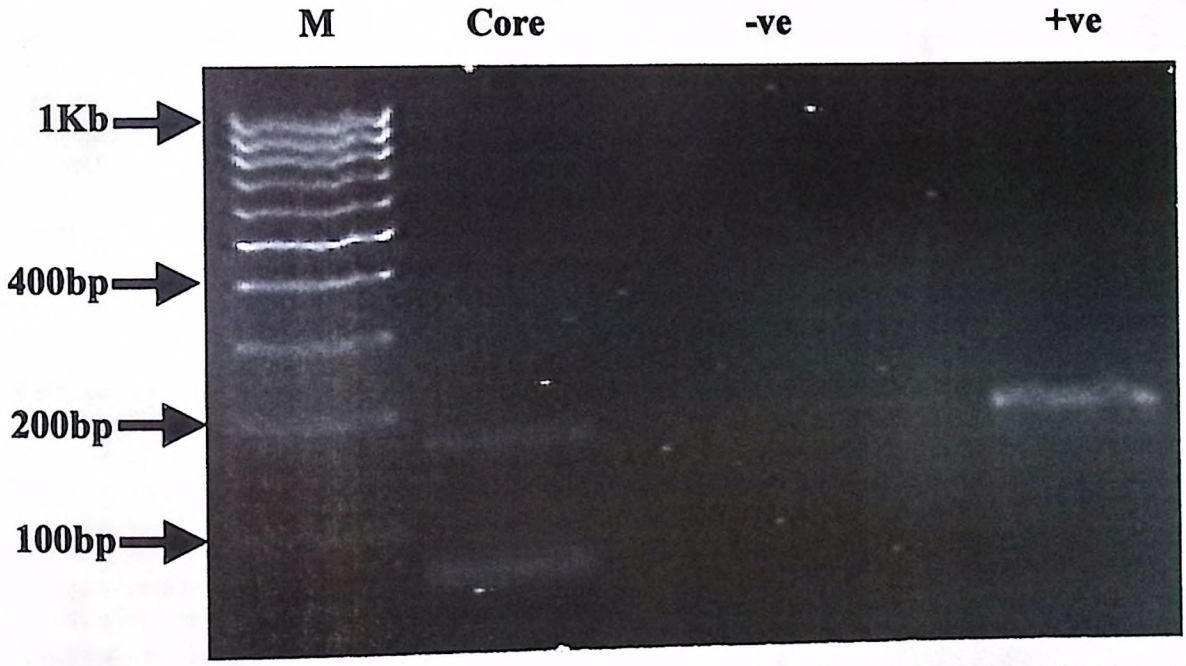


Figure 4.27. Amplification of DNA template from core portion of sambar antler with cytochrome b gene (186 bp).
(M- Molecular marker of 1Kb ladder, –ve = negative control, +ve = positive control).

Table 4.13. Number of attempts undertaken through three different DNA extraction methods of sambar antler.

Target region	Extraction Method		
	Phenol Chloroform	Gene Clean Kit for Ancient Bone DNA	Sodium acetate Precipitation
Core	25	5	12
Cortex	25	5	10
Outer Portion	6	5	6
Basal Portion	4	5	5

Table 4.14. Size and quality of DNA yield from different regions of antler by using sodium acetate method.

Antler region	DNA yield	Remarks
Core	180bp	-
Cortex	<150bp	Degraded
Outer portion	>1kb	-
Basal portion	<150bp	Degraded

```
                                      10        20        30        40        50
                                      |....|....|....|....|....|....|....|....|....|....|
AY540850 Cervus axis                1 TGCTTAATTCTACAAATCCTAACAGGCCTATTCCTAGCAATACACTATAC
AY456912 Cervus axis                1 ........C.............................C..........
AY456911 Cervus axis                1 ........C.....................................
AY456910 Cervus axis                1 ........C.....................................
AY456907 Cervus unicolor            1 ........C.................................G......
AY456906 Cervus unicolor            1 ..TC....C.........A.C.....................
AY456905 Cervus unicolor            1 ..TC....C.........A.C.....................G......
AY456908 Cervus duvaucelii          1 ..TC.............T..C..G.........T..AGC........C..
DQ379303 Cervus duvaucelii          1 ..TC.............T..C..G.........T.............C..
DQ459339 Cervus duvaucelii          1 ..TC.............T..C..G.........T.............C..
DQ459338 Cervus duvaucelii          1 ..TC.............T..C..G.........T.............C..

                                      60        70        80        90        100
                                      |....|....|....|....|....|....|....|....|....|....|
AY540850 Cervus axis               51 ATCCGACACAATAACAGCATTCTCCTCCGTCACCCATATTTGCCGAGACA
AY456912 Cervus axis               51 ...T.....................................................
AY456911 Cervus axis               51 ...T.....................................T...............
AY456910 Cervus axis               51 ...T.....................................T...............
AY456907 Cervus unicolor           51 ..........................................T.........G......
AY456906 Cervus unicolor           51 ......T..............T.....T..T........C........TG
AY456905 Cervus unicolor           51 ..........................................T.........G......
AY456908 Cervus duvaucelii         51 ...T..T..................T..T...........C.........G
DQ379303 Cervus duvaucelii         51 ...T..T..................T..T...........C.........G
DQ459339 Cervus duvaucelii         51 ...T..T..................T..T...........C.........G
DQ459338 Cervus duvaucelii         51 ...T..T..................T..T...........C.........G

                                     110       120       130       140       150
                                      |....|....|....|....|....|....|....|....|....|....|
AY540850 Cervus axis              101 TCAATTATGGCTGAATTATCCGATACATACATGCAAACGGGGCATCAATA
AY456912 Cervus axis              101 ..........G.........T.......C......G.....A..........
AY456911 Cervus axis              101 ...C..................T.......C......G.....A...A.C...
AY456910 Cervus axis              101 ..........G.........T.......Y......G.....A.....C...
AY456907 Cervus unicolor          101 ...G.....................................C.........G......C..T.T
AY456906 Cervus unicolor          101 ................C..T.....T.....C.....................
AY456905 Cervus unicolor          101 ..TC.....................................C.............C..T..
AY456908 Cervus duvaucelii        101 .......C........C.........T..G..C.....T..A..........
DQ379303 Cervus duvaucelii        101 .......C........C.........T..G..C.....T.............
DQ459339 Cervus duvaucelii        101 .......C........C.........T..G..C.....T.............
DQ459338 Cervus duvaucelii        101 .......C........C.........T..G..C.....T.............
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```
                                  160       170       180       190       200
                          ....|....|....|....|....|....|....|....|....|....|
AY540850 Cervus axis      151 TTCTTCATCTGCCTATTTATACATGTAGGACGAGGCCTATATTATGGGTC
AY456912 Cervus axis      151 ..............................GG..................
AY456911 Cervus axis      151 ..............................GG....TT............
AY456910 Cervus axis      151 ..............................GG..................
AY456907 Cervus unicolor  151 ACT.CTC.T..TG..............................C........
AY456906 Cervus unicolor  151 ..T........T...........C..............G..C..C..A..
AY456905 Cervus unicolor  151 ........................G..G..............C........
AY456908 Cervus duvaucelii 151 .....T..T..............................G..C..C..A..
DQ379303 Cervus duvaucelii 151 .....T..Y..............................G..C..C.....
DQ459339 Cervus duvaucelii 151 .....T..T..............................G..C..C.....
DQ459338 Cervus duvaucelii 151 .....T..T..............................G..C..C.....

                                  210       220       230       240       250
                          ....|....|....|....|....|....|....|....|....|....|
AY540850 Cervus axis      201 ATATACTTTCCTAGAAACATGAAACATTGGAGTGATTCTTCTATTCACAG
AY456912 Cervus axis      201 ...C..C.....................C.....A................
AY456911 Cervus axis      201 ...C..C.....................C.....A................
AY456910 Cervus axis      201 ...C..C.....................C.....A................
AY456907 Cervus unicolor  201 N........T.....G...........C.....A................
AY456906 Cervus unicolor  201 ...C.....T.....G...........C.....A..C..C..........
AY456905 Cervus unicolor  201 .........T.....G...........C.....A..C..C..........
AY456908 Cervus duvaucelii 201 ......C..T.....G...............A..C........T....
DQ379303 Cervus duvaucelii 201 ......C..T.....G...........C.....A..C..N.....T....
DQ459339 Cervus duvaucelii 201 ......C..T.....G...............A..C........T....
DQ459338 Cervus duvaucelii 201 ......C..T.....G...............A..C........T....

                                  251 TGATAGCCACAGCTTTTGTAGAATACGTTCCACCATGAGGACAAATATCT
AY540850 Cervus axis      251 .....C.............G.G...T..C.TC.....................
AY456912 Cervus axis      251 ..............................C.T.....................
AY456911 Cervus axis      251 ............T......G........C.......................
AY456910 Cervus axis      251 G....T.......A..C......ACT..C.T.....................A
AY456907 Cervus unicolor  251 .T..........A..C....G...T..C.T.....................A
AY456906 Cervus unicolor  251 .T..........A..C....G...T..C.T.....................A
AY456905 Cervus unicolor  251 .T..........A..C....G...T..C.T.....................A
AY456908 Cervus duvaucelii 251 .T..........A..C....G...T..C.T.....................A
DQ379303 Cervus duvaucelii 251 .T..........A..C....G...T..C.T.....................A
DQ459339 Cervus duvaucelii 251 .T..........A..C....G...T..C.T.....................A
DQ459338 Cervus duvaucelii 251 .T..........A..C....G...T..C.T.....................A

                                  301 TTCTGAGGGGA
AY540850 Cervus axis      301 C..........
AY456912 Cervus axis      301 ...........
AY456911 Cervus axis      301 ...........
AY456910 Cervus axis      301 ...........
AY456907 Cervus unicolor  301 ...........
AY456906 Cervus unicolor  301 ...........
AY456905 Cervus unicolor  301 ...........
AY456908 Cervus duvaucelii 301 ........A.C
DQ379303 Cervus duvaucelii 301 ........A.C
DQ459339 Cervus duvaucelii 301 ........A.C
DQ459338 Cervus duvaucelii 301 ........A.C
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Figure 4.28. Alignment of DNA sequences of cytochrome b region of deer species.

```
                                      10        20        30        40        50
                                ....|....|....|....|....|....|....|....|....|....|
AY391764 Cervus axis          1   GCCTGCCCAGTGACAACCGTTAAACGGCCGCGGTATCCTGACCGTGCAAA
AY391763 Cervus axis          1   ..................................................
AY391762 Cervus axis          1   ..................................................
AY391770 Cervus unicolor      1   ..............G...................................
AY391769 Cervus unicolor      1   ..............G...................................
DQ989296 Cervus duvaucelii    1   ..............G...................................

                                      60        70        80        90       100
                                ....|....|....|....|....|....|....|....|....|....|
AY391764 Cervus axis         51   GGTAGCATAATCACTTGTTCTCTAAATAGGGACTTGTATGAATGGCCACA
AY391763 Cervus axis         51   ..................................................
AY391762 Cervus axis         49   ..................................................
AY391770 Cervus unicolor     51   ..................................................
AY391769 Cervus unicolor     51   ..................................................
DQ989296 Cervus duvaucelii   51   ....................C.............................

                                     110       120       130       140       150
                                ....|....|....|....|....|....|....|....|....|....|
AY391764 Cervus axis        101   CGAGGGTTTTACTGTCTCTTGCTTCCAATCAGTGAAATTGACCTTCCCGT
AY391763 Cervus axis        101   ..................................................
AY391762 Cervus axis         99   ..................................................
AY391770 Cervus unicolor    101   ....................A.............................
AY391769 Cervus unicolor    101   ....................A.............................
DQ989296 Cervus duvaucelii  101   ....................A.............................

                                     160       170       180       190       200
                                ....|....|....|....|....|....|....|....|....|....|
AY391764 Cervus axis        151   GAAGAGGCGGGAATACATTAATAAGACGAGAAGACCCTATGGAGCTTTAA
AY391763 Cervus axis        151   ..................................................
AY391762 Cervus axis        149   ..................................................
AY391770 Cervus unicolor    151   ..............T...................................
AY391769 Cervus unicolor    151   ..............T...................................
DQ989296 Cervus duvaucelii  151   ..............T...................................

                                     210       220       230       240       250
                                ....|....|....|....|....|....|....|....|....|....|
AY391764 Cervus axis        201   CTACTTGACCCAAA GAAATAAATTTCATCGCTAAGGAAACAACAACACT
AY391763 Cervus axis        201   ..............  ..................................
AY391762 Cervus axis        199   ..............  ..................................
AY391770 Cervus unicolor    201   ......AG......  ..G.C.........TA.C................
AY391769 Cervus unicolor    201   ......AG......  ..G.C.........TA.C................
DQ989296 Cervus duvaucelii  201   ......AG......A..............T................T...

                                     260       270       280       290       300
                                ....|....|....|....|....|....|....|....|....|....|
AY391764 Cervus axis        250   CTTCATGGGCCAACAGCTTTGGTTGGGGTGACCTCGGAGAACAAGAAATC
AY391763 Cervus axis        250   ..................................................
AY391762 Cervus axis        248   ..................................................A.
AY391770 Cervus unicolor    250   ...T......T.......................................A.
AY391769 Cervus unicolor    250   ...T......T.....................................A.....
DQ989296 Cervus duvaucelii  251   ...T......T.....................................

                                     310       320       330       340       350
                                ....|....|....|....|....|....|....|....|....|....|
AY391764 Cervus axis        300   CTCCGAGCGATTTTAAAGACTAGACCTACAAGTCGAATCACACAATCGTT
AY391763 Cervus axis        300   ..................................................
AY391762 Cervus axis        298   ..................................................
AY391770 Cervus unicolor    300   ..................................................
AY391769 Cervus unicolor    300   ..................................................
DQ989296 Cervus duvaucelii  301   ..................................................
```

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                                    350        360        370        380        390        400
                                    ....|....|....|....|....|....|....|....|....|....|....|....|
AY391764 Cervus axis          350 TATTGATCCAAAAAA TTGATCAACGGAACAAGTTACCCTAGGGATAACA
AY391763 Cervus axis          350 ...............-....................................
AY391762 Cervus axis          348 ..............A.....................................
AY391770 Cervus unicolor      350 ...............-....................................
AY391769 Cervus unicolor      350 ...............-....................................
DQ989296 Cervus duvaucelii    351 ...............-....................................

                                    410        420        430        440        450
                                    ....|....|....|....|....|....|....|....|....|....|....|....|
AY391764 Cervus axis          399 GCGCAATCCTATTCAAGAGTCCATATCGACAATAGGGTTTACGACCTCGA
AY391763 Cervus axis          399 ..................................................
AY391762 Cervus axis          398 ..................................................
AY391770 Cervus unicolor      399 ..................................................
AY391769 Cervus unicolor      399 ..................................................
DQ989296 Cervus duvaucelii    400 ..................................................

                                    460        470        480        490        500
                                    ....|....|....|....|....|....|....|....|....|....|....|....|
AY391764 Cervus axis          449 TGTTGGATCAGGACATCCCGATGGTGCA CCGCTATCAAAGGTTCGTTTG
AY391763 Cervus axis          449 ...G.....................A.....................
AY391762 Cervus axis          448 ..........................A.....................
AY391770 Cervus unicolor      449 ..........................A.....................
AY391769 Cervus unicolor      449 ..........................A.....................
DQ989296 Cervus duvaucelii    450 ..........................A.....................

                                    510        520        530
                                    ....|....|....|....|....|..
AY391764 Cervus axis          498 TTCA-CGATTAA-GTCCTACGTGATCTGAGTT
AY391763 Cervus axis          499 ....-.......A...................
AY391762 Cervus axis          498 ....A.......A...................
AY391770 Cervus unicolor      499 .C..-.......A...................
AY391769 Cervus unicolor      499 ....-.......A...................
DQ989296 Cervus duvaucelii    500 ....A.......A.................C
```

Figure 4.29. Alignment of DNA sequence of 16S r RNA region of deer species.

Partial sequences of mitochondrial genes cytochrome b of deer species viz. chital, sambar and swamp deer were analysed using 4 sequences of *Axis axis* and *Cervus duvaucelii* and 3 sequences of *Cervus unicolor*. Polymorphism was noticed at 89 different sites out of 311bp of cytochrome b gene (28.62%) of deer species (Fig. 4.28). But, 16S rRNA gene revealed less polymorphism (4.89%) at 26 variable sites out of 532bp (Fig. 4.29).

TspEI
Tru1I AatI BsuRI CviJI
Tru9I Pme55I MaeI BstF5I BsaMI BsmI
tgcttaattctacaaatcctaacaggcctattcctagcaatacactatacatccgacacaataacagcattctcc base pairs
acgaattaagatgtttaggattgtccggataaggatcgttatgtgatatgtaggctgtgttattgtcgtaagagg 1 to 75
MseI StuI Eco147I FokI Mva1269I
Sse9I HaeIII BfaI
Tsp509I PalI SseBI

MaeIII Tsp509I
Tsp45I Sse9I TspEI NspI MwoI
MnlI HphI Alw26I CviJI NlaIII SfaNI
tccgtcacccatatttgccgagacatcaattatggctgaattatccgatacatacatgcaaacggggcatcaata base pairs
aggcagtgggtataaacggctctgtagttaataccgacttaataggctatgtatgtacgtttgccccgtagttat 76 to 150
BsmAI Sse9I Hsp92II SspI
BseRI TspEI Tsp509I

NspI CviJI SseBI
NlaIII AatI Eco147I
MboII BspLU11I Pme55I MaeI NlaIII
ttcttcatctgcctatttatacatgtaggacgaggcctatattatgggtcatatactttcctagaaacatgaaac base pairs
aagaagtagacggataaatatgtacatcctgctccggatataatacccagtatatgaaaggatctttgtactttg 151 to 225
AflIII StuI MnlI BfaI Hsp92II
Hsp92II HaeIII
PalI BsuRI

Hsp92II
XmnI BsiYI
MslI HinfI MboII TspRI CviJI MaeII Bsc4I
attggagtgattcttctattcacagtgatagccacagcttttgtagaatacgttccaccatgaggacaaatatct base pairs
taacctcactaagaagataagtgtcactatcggtgtcgaaaacatcttatgcaaggtggtactcctgtttataga 226 to 300
TfiI CviJI AluI Asp700I BslI
NlaIII
MnlI
DdeI MnlI
ttctgagggga base pairs
aagactcccct 301 to 311
BstDEI

Figure 4.30. Restriction enzyme map of cytochrome b region of *Axis axis*.

CviJI
AatI BsuRI
Pme55I MaeI BstF5I
tgtctaatcctacaaatcatcacaggcctattcctagcaatacactatacatccgatacaataacagcattttcc base pairs
acagattaggatgtttagtagtgtccggataaggatcgttatgtgatatgtaggctatgttattgtcgtaaaagg 1 to 75
StuI Eco147I FokI
HaeIII BfaI
PalI SseBI

Tsp509I TfiI
MnlI Sse9I CviJI TthHB8I MwoI SfaNI
tctgttacccatatctgccgagatgtcaattatggctgaatcattcgatatatacacgcaaacggggcatcaata base pairs
agacaatgggtatagacggctctacagttaataccgacttagtaagctatatatgtgcgtttgccccgtagttat 76 to 150
MaeIII TspEI HinfI TaqI SspI

CviJI SseBI MboI DpnI
BsaAI AatI Eco147I Bsp143I BfaI NlaIII
AflIII Pme55I Csp6I Sau3AI XbaI Alw26I
tttttcatctgtctatttatacacgtaggacgaggcctgtactacggatcatacacttttctagagacatgaaac base pairs
aaaaagtagacagataaatatgtgcatcctgctccggacatgatgcctagtatgtgaaaagatctctgtactttg 151 to 225
MaeII StuI MnlI AfaI Kzo9I MaeI BsmAI
HaeIII RsaI NdeII AlwI Hsp92II
PalI BsuRI DpnII AclWI

BsaMI MnlI
MnlI CviJI .BsmI NlaIII
atcggagtaatcctcctattcacagttatagccacagcattcgtaggatatgtcctaccatgaggacaaatatca base pairs
tagcctcattaggaggataagtgtcaatatcggtgtcgtaagcatcctatacaggatggtactcctgtttatagt 226 to 300
Mva1269I Hsp92II

DdeI MnlI
ttctgagggga base pairs
aagactcccct 301 to 311
BstDEI

Figure 4.31. Restriction enzyme map of cytochrome b region of *Cervus unicolor*.

Tsp509I HaeIII Bst98I
Tsp509I TspEI AsuI PalI MspCI Tru1I BsaMI
Sse9I Sse9I Cfr13I BsuRI BfrI BsmI
tgtctaattctacaaattctcacgggcctattcttaagcatacactacacatctgatacaataacagcattctct base pairs
acagattaagatgtttaagagtgcccggataagaattcgtatgtgatgtgtagactatgttattgtcgtaagaga 1 to 75
TspEI AcsI Sau96I BspTI Tru9I Mva1269I
ApoI AspS9I AflII MseI
CviJI Vha464I

EarI HphI MaeII BsmAI TspEI
Tsp45I BbiII Alw26I HinfI
Eam1104I Hin1I AcyI Sse9I BstF5I Cac8I MwoI SfaNI
tctgtcacccatatctgccgagacgtcaattacggctgaatcatccgatatatgcacgcaaatggagcatcaata base pairs
agacagtgggtatagacggctctgcagttaatgccgacttagtaggctatatacgtgcgtttacctcgtagttat 76 to 150
MaeIII Msp17I Esp3I CviJI FokI MslI SspI
Ksp632I Hsp92I AatII TfiI
MboII BsaHI BsmBI Tsp509I

NspI CviJI SseBI MboI DpnI
NlaIII AatI Eco147I Bsp143I BfaI NlaIII
BspLU11I Pme55I Csp6I Sau3AI XbaI Alw26I
ttctttatttgcctatttatacatgtaggacgaggcctgtactacggatcatataccttttctagagacatgaaac base pairs
aagaaataaacggataaatatgtacatcctgctccggacatgatgcctagtatatggaaagatctctgtactttg 151 to 225
AflIII StuI MnlI AfaI Kzo9I MaeI BsmAI
Hsp92II HaeIII RsaI NdeII AlwI Hsp92II
PalI BsuRI DpnII AclWI

BsaMI MnlI
CviJI BsmI NlaIII
attggagtaatccttctatttacagttatagccacagcattcgtaggatatgtcctaccatgaggacaaatatca base pairs
taacctcattaggaagataaatgtcaatatcggtgtcgtaagcatcctatacaggatggtactcctgtttatagt 226 to 300
Mva1269I Hsp92II

DdeI MnlI
ttctgagggga base pairs
aagactcccct 301 to 311
BstDEI

Figure 4.32. Restriction enzyme map of cytochrome b region of *Cervus duvauceli*.

Table 4.15. Restriction enzymes cutting partial fragments of cytochrome b gene among deer species.

Enzyme	Recognition sequence	*Axis axis*		*Cervus unicolor*		*Cervus duvauceli*	
		No. of cuts	Positions of sites	No. of cuts	Positions of sites	No. of cuts	Positions of sites
AluI	**ag/ct**	**1**	**262**	-	-	-	-
Alw26I	gtctc	1	99	1	218	2	99 218
Asp700I	gaann/nnttc	1	275	-	-	-	-
AatII	gacgt/c	-	-	-	-	1	101
AcsI	r/aatty	-	-	-	-	1	14
AcyI	gr/cgyc	-	-	-	-	1	98
AflII	c/ttaag	-	-	-	-	1	33
ApoI	r/aatty	-	-	-	-	1	14
AspS9I	g/gncc	-	-	-	-	1	24
AsuI	g/gncc	-	-	-	-	1	24
Bsc4I	**ccnnnn/nnng**	**1**	**285**	-	-	-	-
BsaAI	yac/gtr	-	-	1	174	-	-
BbiII	gr/cgyc	-	-	-	-	1	98
BseRI	**gaggag**	**1**	**77**	-	-	-	-
BsiYI	**ccnnnnn/nng**	**1**	**286**	-	-	-	-
BslI	**ccnnnnn/nng**	**1**	**286**	-	-	-	-
Bsp143I	/gatc	-	-	1	196	-	-
BstDEI	c/tnag	-	-	1	303	-	-
BfrI	c/ttaag	-	-	-	-	1	33
BsaHI	gr/cgyc	-	-	-	-	1	98
BsmBI	cgtctc	-	-	-	-	1	100
BsuRI	gg/cc	-	-	-	-	2	185
Bsp143I	/gatc	-	-	-	-	1	196
BspTI	c/ttaag	-	-	-	-	1	33
Bst98I	c/ttaag	-	-	-	-	1	33
BstF5I	ggatg	-	-	-	-	1	121
Cac8I	gcn/ngc	-	-	-	-	1	131
Cfr13I	g/gncc	-	-	-	-	1	24
Eam1104I	ctcttc	-	-	-	-	1	77
EarI	ctcttc	-	-	-	-	1	77
Esp3I	cgtctc	-	-	-	-	1	100
Hsp92II	catg/	4	133 175 221 287	2	221 287	3	175 221 287
Hsp92I	gr/cgyc	-	-	-	-	1	98
HinlI	gr/cgyc	-	-	-	-	1	98
Ksp632I	ctcttc	-	-	-	-	1	77
MaeII	a/cgt	1	275	1	173	1	98
MboII	**gaaga**	**2**	**156 241**	-	-	**1**	77

MboI	/gatc	-	-	-	-	1	196
MnlI	cctc	4	77 185 290 308	5	77 185 240 290 308	3	185 290 308
MseI	t/taa	1	4	-	-	1	34
MslI	**caynn/nnrtg**	**1**	**229**	-	-	-	-
MslI	caynn/nnrtg	-	-	-	-	1	134
Msp17I	gr/cgyc	-	-	-	-	1	98
MspCI	c/ttaag	-	-	-	-	1	33
Mva1269I	gaatgc	1	72	1	267	2	72 267
NlaIII	catg/	4	133 175 221 287	2	221 287	3	175 221 287
NspI	rcatg/y	2	133 175	-	-	1	175
Sse9I	/aatt	3	102 113	1	102	3	5 14 102
Sau96I	g/gncc	-	-	-	-	1	24
Tru1I	t/taa	1	4	-	-	1	34
TaqI	t/cga	-	-	1	120	-	-
Tru9I	t/taa	1	4	-	-	1	34
		-	-	-	-	-	-
Tsp509I	/aatt	3	102 113	1	102	3	5 14 102
TspEI	/aatt	3	102 113	1	102	3	5 14 102
TspRI	cagtg	1	252	-	-	-	-
TthHB8I	t/cga	-	-	1	120	-	-
Vha464I	c/ttaag	-	-	-	-	1	33
XmnI	**gaann/nnttc**	**1**	**275**	-	-	-	-

Restriction enzymes which cut the cytochrome b gene fragment at different sites in deer species have been listed in Table 4.15 and Fig. 4.30 to 4.32. These can be used to differentiate species from one another. Eight restriction enzymes were found specific to *Axis axis* DNA fragments, where as 5 restriction enzymes were specific to *Cervus unicolor* and 30 to *Cervus duvauceli*. There were 9 restriction enzymes which are common to all three species but, cuts at variable positions.

Bse1I BslI XmaIII BsuRI BseDI ThaI MspA1I AciI MwoI
BsrSI MseI EaeI Eco52I BsiEI DsaI AccII KspI SacII
Cac8I Tsp45I TspRI Bsc4I EagI PalI Bsh1285I BsaOI Bsh1236I BslI
gcctgcccagtgacaaccgttaaacggccgcggtatcctgaccgtgcaaaggtagcataatcacttgttctctaa base pairs
cggacgggtcactgttggcaatttgccggcgccataggactggcacgtttccatcgtattagtgaacaagagatt 1 to 75
MaeIII Tru9I BstZI CviJI Fsp4HI ItaI MvnI SstII Bsc4I
BseNI Tru1I CfrI HaeIII BsaJI BstMCI NspBII Cfr42I
BsrI BsiYI EclXI BsoFI BstDSI BstUI Sfr303I BsiYI

PalI BalI MnlI
HaeIII BslI
TspEI
BsmFI EaeI MscI BsiI Alw26I TspRI
atagggacttgtatgaatggccacacgagggttttactgtctcttgcttccaatcagtgaaattgaccttcccgt base pairs
tatccctgaacatacttaccggtgtgctcccaaaatgacagagaacgaaggttagtcactttaactggaagggca 76 to 150
CfrI MluNI BssSI BsmAI Sse9I
CviJI Bsc4I Tsp509I
BsuRI BsiYI

Eam1104I AseI BpiI Tru1I Tsp509I
BsiYI AciI Tru9I Bbv16II Tru9I TspEI
Bsc4I EarI AsnI Tru1I MboII CviJI Sse9I
gaagaggcgggaatacattaataagacgagaagaccctatggagctttaactacttgacccaaagaaataaattt base pairs
cttctccgcccttatgtaattattctgctcttctgggatacctcgaaattgatgaactgggtttctttatttaaa 151 to 225
BslI MnlI VspI BpuAI AluI AcsI
MboII FauI PshBI BbsI MseI ApoI
Ksp632I MseI

Cfr13I HaeIII PspEI HphI
EarI Hsp92II BsuRI BstPI MaeIII
DdeI Eam1104I AspS9I AluI Eco91I BsaJI
catcgctaaggaaacaacaacactcttcatgggccaacagctttggttggggtgacctcggagaacaagaaatcc base pairs
gtagcgattcctttgttgttgtgagaagtacccggttgtcgaaaccaaccccactggagcctcttgttctttagg 226 to 300
BstDEI Ksp632I AsuI PalI BstEII BseDI
MboII Sau96I CviJI EcoO65I MnlI
NlaIII CviJI Tsp45I

Bsp143I TspEI MboI DpnI
Tru1I TaqI MboI DpnI BclI Bsp143I
MnlI Tru9I MaeI DrdI HinfI DpnII AclWI FbaI Sau3AI
tccgagcgattttaaagactagacctacaagtcgaatcacacaatcgtttattgatccaaaaaattgatcaacgg base pairs
aggctcgctaaaatttctgatctggatgttcagcttagtgtgttagcaaataactaggtttttaactagttgcc 301 to 375
MseI BfaI TthHB8I NdeII AlwI DpnII Kzo9I
DraI TfiI Sau3AI Sse9I NdeII
Kzo9I Tsp509I Ksp22I

StyI EcoT14I AspLEI
MnlI
Eco130I Hin6I HinfI TthHB8I TthHB8I
MaeIII BssT1I BfaI HinP1I
aacaagttaccctagggataacagcgcaatcctattcaagagtccatatcgacaatagggtttacgacctcgatg base pairs
ttgttcaatgggatccctattgtcgcgttaggataagttctcaggtatagctgttatcccaaatgctggagctac 376 to 450
AvrII MaeI HspAI PleI TaqI TaqI
ErhI BsaJI HhaI

BlnI BseDI CfoI

Bsp143I Bsp1286I MboI DpnI
MboI DpnI FokI VneI BmyI AciI TruI1 DpnII DdeI
DpnII AclWI BstF5I SduI BsiHKAI Tru9I MaeII Bsp143I
ttggatcaggacatcccgatggtgcaccgctatcaaaggttcgtttgttcacgattaagtcctacgtgatctgag base pairs
aacctagtcctgtagggctaccacgtggcgatagtttccaagcaaacaagtgctaattcaggatgcactagactc 451 to 525
NdeII AlwI MslI Alw44I Alw21I MseI BsaAI Kzo9I
Sau3AI ApaLI Bbv12I NdeII BstDEI
Kzo9I AspHI Sau3AI

tt base pairs
aa 526 to 527

Figure 4.33. Restriction enzyme map of *Axis axis* 16S r RNA gene generated using Webcutter 2.

Bse1I BsaOI Bsc4I XmaIII BsuRI BseDI ThaI MspA1I SacII
BsrSI Bsh1285I BsiYI Eco52I BsiEI DsaI AccII KspI AciI MwoI
Cac8I Tsp45I TspRI MseI BstZI HaeIII Fsp4HI ItaI MvnI SstII Bsc4I
gcctgcccagtgacgaccgttaaacggccgcggtatcctgaccgtgcaaaggtagcataatcacttgttctctaa base pairs
cggacgggtcactgctggcaatttgccggcgccataggactggcacgtttccatcgtattagtgaacaagagatt 1 to 75
MaeIII BsiEI TruI1 CfrI CviJI BsaJI BstMCI NspBII Cfr42I
BseNI BstMCI BslI EagI PalI Bsh1285I BsaOI Bsh1236I BslI
BsrI Tru9I EaeI EclXI BsoFI BstDSI BstUI Sfr303I BsiYI

PalI BalI MnlI
HaeIII BslI TspEI
BsmFI EaeI MscI BsiI Alw26I TspRI
atagggacttgtatgaatggccacacgagggttttactgtctcttacttccaatcagtgaaattgaccttcccgt base pairs
tatccctgaacatacttaccggtgtgctcccaaaatgacagagaatgaaggttagtcactttaactggaagggca 76 to 150
CfrI MluNI BssSI BsmAI Sse9I
CviJI Bsc4I Tsp509I
BsuRI BsiYI

Eam1104I AseI BpiI TruI1 TspEI
BsiYI AciI Tru9I Bbv16II Tru9I CviJI Sse9I
Bsc4I EarI AsnI TruI1 MboII CviJI DdeI Alw26I
gaagaggcgggaatatattaataagacgagaagaccctatggagctttaactacttagcccaaagagacaaattt base pairs
cttctccgcccttatataattattctgctcttctgggatacctcgaaattgatgaatcgggtttctctgtttaaa 151 to 225
BslI MnlI VspI BpuAI AluI BstDEI BsmAI
MboII FauI PshBI BbsI MseI AcsI

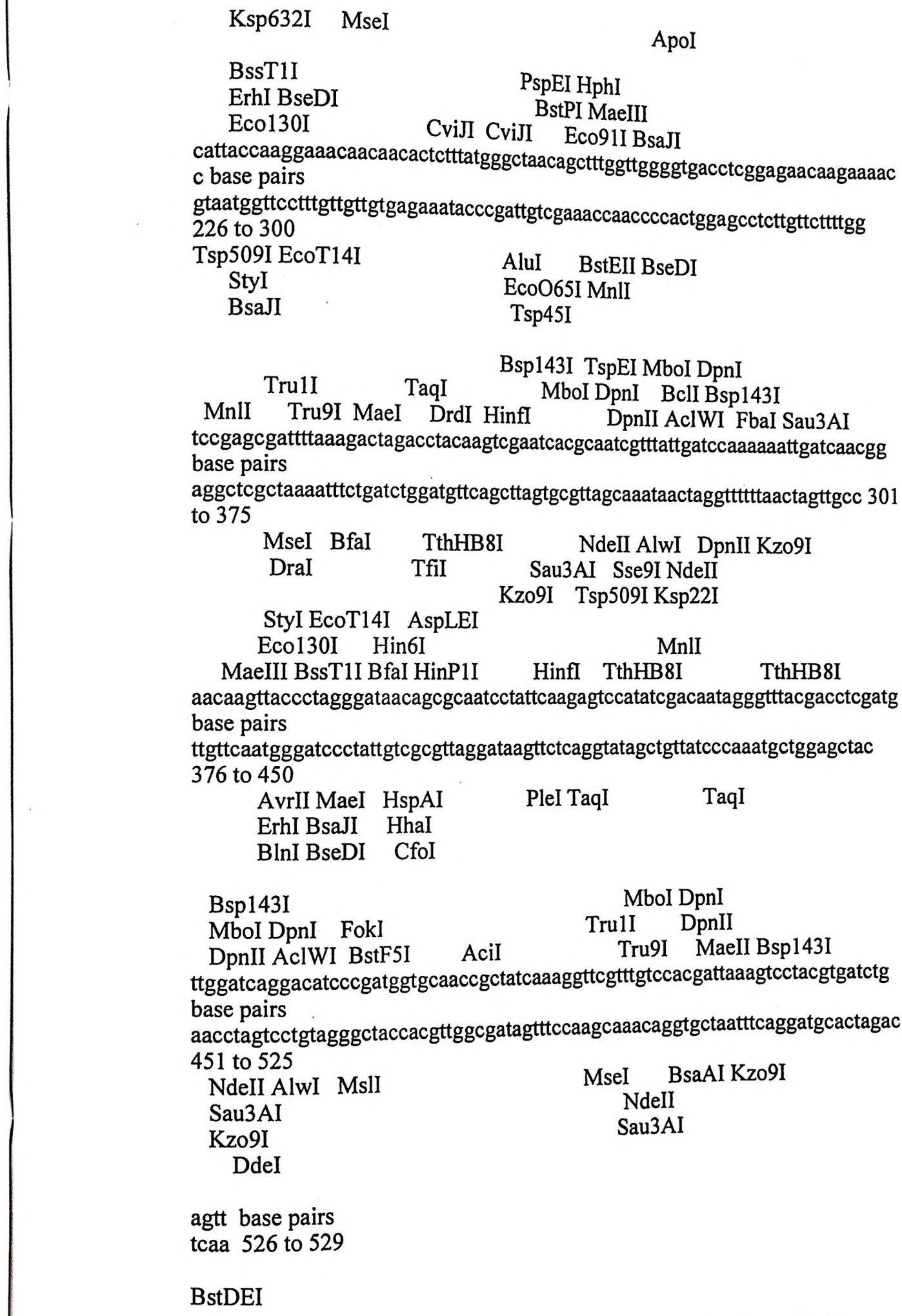

Figure 4.34. Restriction enzyme map of *Cervus unicolor* 16S r RNA gene generated using Webcutter 2.

Bse1I BsaOI Bsc4I XmaIII BsuRI BseDI ThaI MspA1I SacII
BsrSI Bsh1285I BsiYI Eco52I BsiEI DsaI AccII KspI AciI MwoI
Cac8I Tsp45I TspRI MseI BstZI HaeIII Fsp4HI ItaI MvnI SstII Bsc4I
gcctgcccagtgacgaccgttaaacggccgcggtatcctgaccgtgcaaaggtagcataatcacttgttcccta
a base pairs
cggacgggtcactgctggcaatttgccggcgccataggactggcacgtttccatcgtattagtgaacaagggatt
1 to 75
MaeIII BsiEI Tru1I CfrI CviJI BsaJI BstMCI NspBII Cfr42I
BseNI BstMCI BslI EagI PalI Bsh1285I BsaOI Bsh1236I BslI
BsrI Tru9I EaeI EclXI BsoFI BstDSI BstUI Sfr303I BsiYI

PalI BalI MnlI
BsiYI HaeIII BslI TspEI
Bsc4I BsmFI EaeI MscI BsiI Alw26I TspRI
atagggacttgtatgaatggccacacgagggttttactgtctcttacttccaatcagtgaaattgaccttcccgt
base pairs
tatccctgaacatacttaccggtgtgctcccaaaatgacagagaatgaaggttagtcactttaactggaagggca
76 to 150
BslI CfrI MluNI BssSI BsmAI Sse9I
CviJI Bsc4I Tsp509I
BsuRI BsiYI

Eam1104I AseI BpiI Tru1I Tsp5
BsiYI AciI Tru9I Bbv16II Tru9I CviJI TspEI
Bsc4I EarI AsnI Tru1I MboII CviJI DdeI Sse9I
gaagaggcgggaatatattaataagacgagaagaccctatggagctttaactacttagcccaaaagaaataaatt
base pairs
cttctccgcccttatataattattctgctcttctgggatacctcgaaattgatgaatcgggttttctttatttaa 151 to
225
BslI MnlI VspI BpuAI AluI BstDEI AcsI
MboII FauI PshBI BbsI MseI ApoI
Ksp632I MseI

09I PspEI HphI
BstDEI BstPI MaeIII
DdeI CviJI CviJI Eco91I BsaJI
tcattgctaaggaaacaacaatactctttatgggctaacagctttggttggggtgacctcggagaacaaaaaatc
base pairs
agtaacgattcctttgttgttatgagaaatacccgattgtcgaaaccaaccccactggagcctcttgtttttag
226 to 300
BsrDI AluI BstEII BseDI
EcoO65I MnlI
Tsp45I

Bsp143I TspEI MboI DpnI
MboI DpnI BclI Bsp143I
Tru1I TaqI DpnII AclWI FbaI Sau3AI
MnlI Tru9I MaeI DrdI HinfI
ctccgagcgattttaaagactagacctacaagtcgaatcacacaatcgcttattgatccaaaaaattgatcaacg
base pairs
gaggctcgctaaaatttctgatctggatgttcagcttagtgtgttagcgaataactaggttttttaactagttgc 301

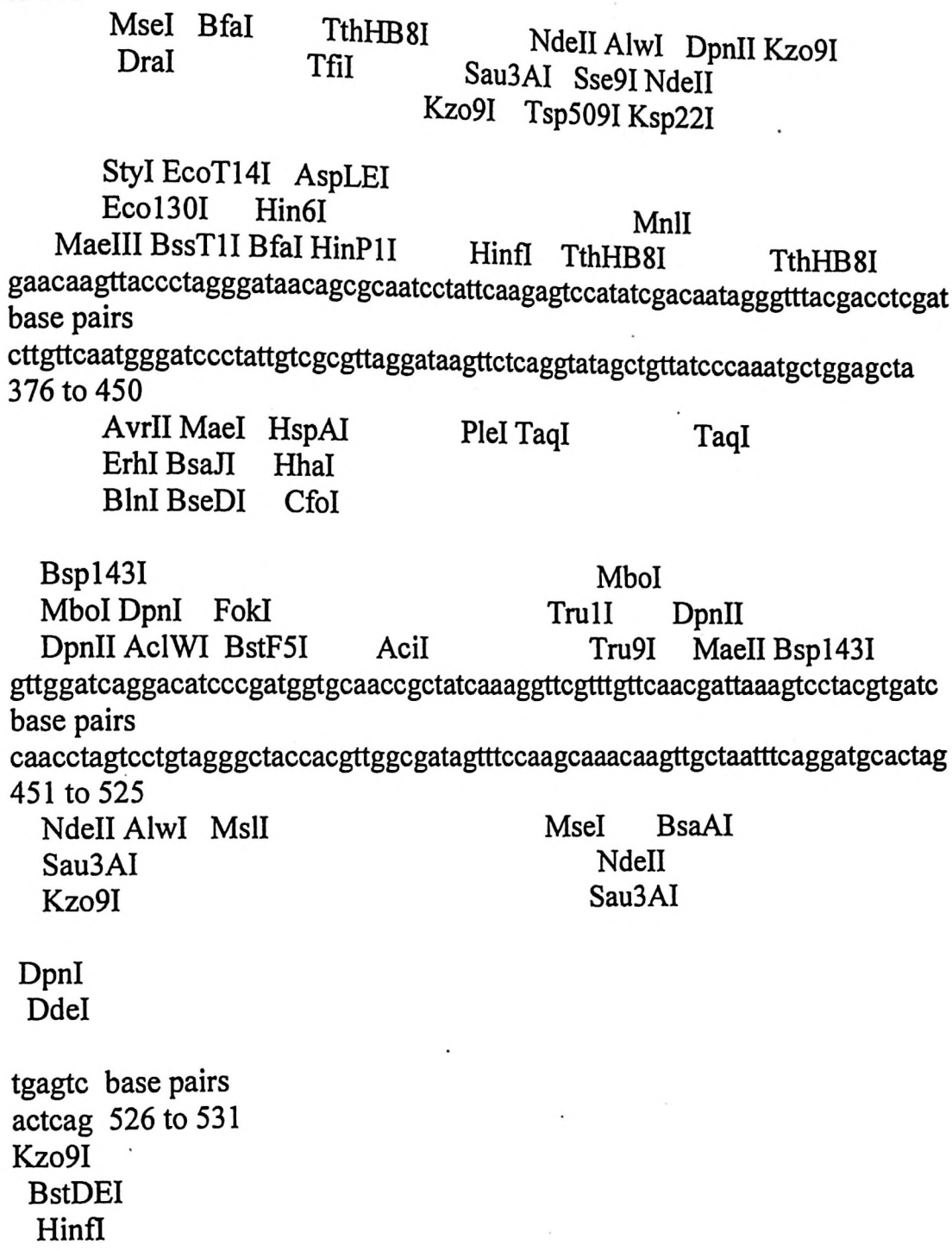

Figure 4.35. Restriction enzyme map of *Cervus duvauceli* 16S r RNA gene generated using Webcutter 2.

Table 4.16. Restriction enzymes cutting partial fragment of 16S rRNA gene of deer species.

Enzyme	Recognition sequence	*Axis axis*		*Cervus unicolor*		*Cervus duvauceli*	
		No. of cuts	Positions of sites	No. of cuts	Positions of sites	No. of cuts	Positions of sites
Alw21I	**gwgcw/c**	**1**	**476**	-	-	-	-
Alw44I	**g/tgcac**	**1**	**472**	-	-	-	-
ApaLI	**g/tgcac**	**1**	**472**	-	-	-	-
AspHI	**gwgcw/c**	**1**	**476**	-	-	-	-
AspS9I	**g/gncc**	**1**	**256**	-	-	-	-
AsuI	**g/gncc**	**1**	**256**	-	-	-	-
Bbv12I	**gwgcw/c**	**1**	**476**	-	-	-	-
BmyI	**gdgch/c**	**1**	**476**	-	-	-	-
BsaAI	**yac/gtr**	**1**	**515**	-	-	-	-
BsaJI	c/cnngg	3	28 281 386	4	28 231 281 386	3	28 282 387
BseDI	c/cnngg	3	28 281 386	4	28 231 281 386	3	28 282 387
BsiHKAI	**gwgcw/c**	**1**	**476**	-	-	-	-
Bsp1286I	**gdgch/c**	**1**	**476**	-	-	-	-
Bsp143I	/gatc	4	353 366 453 517	4	353 366 453 519	4	354 367 454 521
BssT1I	c/cwwgg	1	386	2	231 386	1	387
BstDEI	c/tnag	2	231 521	2	204 523	3	204 232 525
BsaAI	yac/gtr	-	-	1	517	1	519
BsrDI	gcaatg	-	-	-	-	1	232
Cfr13I	**g/gncc**	**1**	**256**	-	-	-	-
CviJI	rg/cy	5	27 95 194 258 265	6	27 95 194 208 258 265	6	27 95 194 208 259 266
DdeI	c/tnag	2	231 521	2	204 523	3	204 232 525
DpnI	ga/tc	4	355 368 455 519	4	355 368 455 521	4	356 369 456 523
DpnII	/gatc	4	353 366 453 517	4	353 366 453 519	4	354 367 454 521
Eco130I	c/cwwgg	1	386	2	231 386	1	387
EcoT14I	c/cwwgg	1	386	2	231 386	1	387
ErhI	c/cwwgg	1	386	2	231 386	1	387

Hsp92II	catg/	1	256	-	-	-	-
Kzo9I	/gatc	4	353 366 453 517	4	353 366 453 519	4	4 354 367 454 521
MaeII	a/cgt	1	514	1	516	1	518
MboI	/gatc	4	353 366 453 517	4	353 366 453 519	4	354 367 454 521
MnlI	cctc	5	105 157 284 302 446	5	105 157 284 302 446	5	105 157 285 303 447
MseI	t/taa	5	20 168 197 312 505	5	20 168 197 312 506	5	20 168 197 313 508
NdeII	/gatc	4	353 366 453 517	4	353 366 453 517	4	354 367 454 521
NlaIII	**catg/**	**1**	**256**	-	-	-	-
Sau96I	**g/gncc**	**1**	**256**	-	-	-	-
SduI	**gdgch/c**	**1**	**476**	-	-	-	-
Sfr303I	**ccgc/gg**	**1**	**31**	-	-	-	-
StyI	c/cwwgg	1	386	2	231 386	1	387
TruII	t/taa	5	20 168 197 312 505	5	20 168 197 312 506	5	20 168 197 313 508
Tru9I	t/taa	5	20 168 197 312 505	5	20 168 197 312 506	5	20 168 197 313 508
VneI	**g/tgcac**	**1**	**472**	-	-	-	-

Restriction enzymes which were cutting the 16S rRNA gene fragment at different sites in deer species have been listed in Table 4.16 and Fig. 4.33 to 4.35. These can be used to differentiate species from one another. Seventeen restriction enzymes are specific to *Axis axis* DNA fragments, where as only one to *Cervus duvauceli* and none of the restriction enzyme was specific to *Cervus unicolor*. There are 21 restriction enzymes which are common to all three species but, cuts at variable positions.

Universal primers of 250bp fragments were developed using software, Primer 3 of cytochrome b gene (Table 4.17) and similarly 234-249bp fragments primer for 16S rRNA (Table 4.18).

Table 4.17. Primers developed for deer species using Primer 3 software for cytochrome b region.

Primer		Fragment size	Tm	GC contents
Forward	Reverse			
CGACACAATAAC AGCATTCTCCT	GAAAGATATTTGT CCTCATGGTG	250	59.8	41.3
GACACAATAACA GCATTCTCCTC	GAAAGATATTTGT CCTCATGGTG	249	52.7	41.3
GAAAGATATTTG TCCTCATGGTG	AGAAAGATATTT GTCCTCATGGTG	250	58.2	40.5
CGACACAATAAC AGCATTCTCCTC	GAAAGATATTTGT CCTCATGGTG	250	59.4	42.5

Table 4.18. Primers developed for deer species using Primer 3 software for 16S rRNA region.

Primer		Fragment size	Tm	GC contents
Forward	Reverse			
GACCGTGCAAAG GTAGCATAA	GAGGTCACCCCA ACCAAAG	245	60.3	52.8
GACCGTGCAAAG GTAGCATA	GAGGTCACCCCA ACCAAAG	245	59.5	53.9
TCCTGACCGTGC AAAGGTA	GAGGTCACCCCA ACCAAAG	249	60.3	55.2
ATCCTGACCGTG CAAAGGTA	AAGCTGTTGGCCC ATGAAG	234	60.3	51.3

4.6. Discussion

Morphometry is the best method to differentiate antler of different species when intact. Caro *et al.* (2003) also mentions that morphological features of antlers in Cervidae often used as a diagnostic tool to identify species. Bubenik (1968) reported that antlers are useful in visual characterization, distinguishing sex, species and individual.

Antlers of different species have their unique branching patterns, which helps in distinguishing antler of one species from other. Antlers of these species when intact are easy to differentiate based on branching patterns, numbers of tines, curvature, angles (Fig. 4.6 and 4.8), and roughness. Shed antler will not have cut marks and it will have rough bony surface on underneath the basal portion (Fig. 4.3). Unshed antler is possible to be seized if the animal had an unnatural death, and it might be due to killing by predator, poaching or disease. Velvet antlers have skin covering like, velvet when the antler is still in growth phase (Fig. 4.4). Bubenik (1968), also reports that the antlers are formed by the calcification, beneath the velvet. Inter-specific variations were noticed in branching pattern of antlers (Fig.4.5).

Number of tines is specific to species and helpful in distinguishing antlers of different species. Antler of swamp deer had more than two tines, barking deer had only one tine and others like chital, sambar and hog deer had two tines each. Only second tine of hog deer points downward whereas other tines of all the other four deer species and first tine of hog deer points upward. The above feature of hog deer antler can be diagnostic for its antler and may be differentiated it from antlers of chital and sambar. Among chital and sambar antlers, the dichotomy of second tine of chital antler occurs at different portion of main beam and this feature can be used to differentiate antlers of chital from sambar.

Differences were noted in the curvature of main beam of different deer species (Fig 4.5). In antler of swamp deer, the main beam was too much away from the first tine, in chital the main beam was slanted towards the first tine. Sambar and hog deer

have almost straight main beam. The curvature can support in differentiation when used with other parameters. Geist (1987) noted considerable individual differences in antler sizes, number of ridges and extent of curvature. Bubenik (1968) also mentions about difference in shape and colouration of antlers. Colour of the antler was not used for distinction of species. Texture could probably be used for distinction as mostly the antler of sambar is roughest followed by chital, hog deer and swamp deer.

The number of angles depends on the presence of number of tines. The first angle in Fig. 4.6 indicate that swamp deer antler has widest mean angle measurement (109.25±1.87), followed by chital (103.17±1.66), sambar (65.59±1.87). Hog deer and barking deer antlers had overlapping first mean angle measurements of 51±1.76 and 56.83±6.34 respectively. Figure 4.7 illustrate the second angle of antlers of above mentioned five species. The barking deer antler does not have second tines and angle. Second mean angle measurements were observed to be widest in swamp deer antler (106.20±2.97) followed by hog deer (85.26±2.18), sambar (58.12±3.11) and chital (54.35±2.06). The branching pattern can substantiate wherever overlaps in angle was observed. Histograms of angles indicate about the measurements in various deer species (Fig. 4.7 and 4.9). Confidence interval for the first angle measurements of antlers of four species were different without any overlap hence this variable can be used to differentiate antlers of various species (Fig. 4.10). Three species antlers (chital, sambar and hog deer) had overlapping confidence interval in the second angle measurements, only swamp deer had no overlap. Hence this variable may not be used to differentiate antlers of various species, but when substantiate with branching pattern as well as first angle measurements this may be useful. In Fig. 4.11 the angle measurements of first versus second angles were plotted, however few overlaps were noticed. These might be due to the age effect. These overlaps can be corrected by using physical parameters like branching patterns and number of tines etc.

Discriminant Function analysis of the quantitative variables of morphometric measurements was also useful in differentiating antler of different species. Three functions derived using ten variables could absolutely differentiate the antlers of different species using Discriminant Function Analysis (DFA). First function could

itself explain 57.3% variability (Table 4.4, 4.5 & 4.6). Canonical Discriminant function could cluster antler of different species with few overlaps due to antlers of various age groups (Fig. 4.12). DFA can be used for distinguishing antlers of different species along with the branching patterns. Morphometry only becomes problematic in case of antler of young animals, malformed antler and when antler is broken. In such cases other analytical techniques can be used for inter-specific distinction of antlers. Differentiation in cross-sections of antler of various species can be used for identification (Fig. 4.13).

Scanning electron microscopy (SEM) can also be used successfully to differentiate antler into different species. Even different portions of antler of different species can be distinguished. Outer portion of antler (Fig. 4.14) suffers with environmental impact so, it is better to use cortex and core area for differentiation (Fig 4.15 and 4.16). The surface topography of core portion gave best distinction. No study seems to distinguish antlers based on SEM. Although, few studies viz., Banks and Newberry (1982) reported that the developing antler is composed of an aggregate of distinct cell types including fibroblasts, chondroblast, chondrocytes and osteocytes. Fenessy and Suttie (1985) reported that the tip of velvet antler has few layers of mesenchymal cells which abruptly differentiate to cartilaginous tissue, which are afterwards replaced by bone. Speer (1983) observed that the histology of antler using polarized microscope and reported that longitudinal section showed the presence of epidermis, dermis, peristomium having coarse collagen fibers followed by fine collagen fibers and spongy cells in the center. Antler bone is considered to be one of the toughest bones (Zioupos *et al.* 1994; Vashishth 2004). The outer cylinder of compact antler bone coupled with the spongy core imparts maximal strength to the antler (Goss 1983).

Antler sample matches with hydroxyapatite minerals (American Standard Test Matching file no. 9, card no. – 432). Since antler and ivory powder both constitute of hydroxyapatite minerals, it is important to make distinction between them because a confusion may arise among items of these two species. Minute but distinct differences between ivory and antler diffractogram were observed that is after 50^0. In antler

humps was noticed where as in ivory there was slanting line which indicates more amorphous nature of antler. Crystallite size of ivory ranged between ~ 16.9 – 86.1 nm where as antler ranged between ~ 26.5 –169.8 nm. Chipra and Bish (1991) observed crystallite size of ~ 20 – 65 nm in seismosaurus bone and ~ 8 – 20 nm in elk bone. Cell parameter 'a', 'c' and cell volume was higher in antler (11.70, 6.92 and 820.22) than ivory (9.49, 6.87 and 535.80). Scatter plot between maximum and minimum background intensity could separate antler from ivory in different clusters (Fig.4.17).

All deer species had similar XRD pattern matching with hydroxyapatite. There were minute differences in the diffractogram of these species but, these differences were however not consistent. In diffractogram of chital, a very steep decline was noticed after 50^0 which was not observed in diffractogram of any other deer species. In swamp deer the main peak at 31^0 had shoulder peaks in some samples and had well developed continuous peak after 46^0. Barking deer had small peak at 35^0 which is not present in any other species. In most of the samples of hog deer, there were shoulder peak adjacent to 40^0. In sambar no such distinct feature was noted except well developed peak at 31^0 (Fig.4.18). All species of deer studied had peak at 25^0, 32^0, 46^0, and 53^0. Swamp deer had maximum number of peaks and an extra peak in few samples of swamp deer was characteristics at 32^0. Some of the peaks were only present in swamp deer like 34^0 and 44^0. Barking deer only missed the peaks at 26^0, 49^0. Only chital did not have any peak at 39^0. Sambar had extra peak at 42^0 (Table 4.8). Swamp deer, hog deer and barking deer had peaks at 28^0 but, chital and sambar had peak at 29^0. Dendrogram generated based on cluster analysis using presence and absence of diffracted peaks at different angles differentiated the antler of different species (Fig 4.19).

Fig. 4.20 indicates that the nature of weight loss in antlers of different species were different. Maximum losses were noticed in antlers of all species between 200 and 400^0C. Weight loss pattern of antlers of different species showed remarkable differences in three temperature sections, 0-300^0C, 450-700^0C and 1000-1400^0C. Weight loss in sambar antler is very distinct from other species antlers in all three

temperature sections. Data analysis of antlers in Fig.4.21 indicated variation in percentage water loss at 100^0C and the percentage water loss pattern was highest in chital (7.5%) and lowest in antler of swamp deer (2.5%). Total percentage weight left till 1400 ^{0}C was highest in antlers of hog deer (58%) and lowest in barking deer (43.1%). More samples will be able to give better differentiation among antlers of deer species. Thermographs of antlers of different species in Fig. 4.22 indicate minute but distinct differences. There was endothermic peak at 770-810 ^{0}C in antler of chital, where as three consecutive exothermic peaks at 884 ^{0}C, 1120 ^{0}C and 1339 ^{0}C was observed in the antler of sambar. In antler of swamp deer two peaks were noticed between 800 ^{0}C and 1135 ^{0}C. There was slight difference in thermographs of barking and hog deer, only a minute exothermic peak was noticed in barking deer at 150 ^{0}C. These minute distinct differences may be successfully used as species specific signature.

Antlers are composed primarily of minerals, protein, carbohydrates and fatty acids (Sunwoo *et al.* 1995). Minerals observed in antler are calcium, potassium, chlorine, sulphur, phosphorous, silicon, magnesium, sodium, nitrogen, manganese, iron, copper and zinc (http://www.brunnerbiz.com/ activelife/activelvet.html). Mineral required by herbivores are derived from plant tissues but deposition of minerals in plants is limited by quality of the soils and the underlying geology of the region (Van Soest 1994).

The qualitative elemental fluorescence X-ray scan obtained for antlers of five species reveal that the intensity (KCps) of calcium (Ca) was highest in all species out of the 16 elements analyzed. Major intensity was of calcium (Ca), phosphorous (P), strontium (Sr), iron (Fe), silicon (Si), sodium (Na) and magnesium (Mg) and minor intensity of sulphur (S), zinc (Zn), chlorine (Cl), copper (Cu), aluminum (Al) and potassium (K). Chromium (Cr), nickel (Ni) manganese (Mn), and rare earth elements gadolinium (Gd) and osmium (OS) were in traces (Fig 4.23). Prominent differences among antlers of deer species were noted in the intensities of calcium, phosphorous, sulphur, chlorine, aluminium, zinc, potassium, chromium, manganese, and gadolinium and whereas, intensity of magnesium, copper, nickel, and osmium were

less variable (Fig. 4.23). XRF output indicates that the calcium and phosphorous ratios were constant (approx. 2:1) (Fig.4.23a) in antler of all deer species. Chapman (1975) and Moen *et al.* (1999) has also reported a similar ratio of calcium and phosphorus.

Distinctly high intensity of silicon, aluminum, magnesium, potassium and zinc in antler of sambar, iron and chromium in swamp deer, sulphur and chlorine in chital, potassium in barking deer and strontium and aluminum in hog deer may be useful in characterizing antlers of these species (Fig. 4.23). Intensity of gadolinium was only detected in sambar and hog deer (Fig. 4.23 e). It is not necessary that if XRF intensity of an element is high its concentration will also be high. Although sample size were low but, higher intensity in some antler and lower intensity in other antler of various elements and presence and absence of intensities of few elements can be used for inter-specific distinction.

The mean elemental concentrations of antlers of various species indicates that the major constituent was calcium (95% in antlers of swamp deer, 94% in antlers of chital, sambar and hog deer and 92% in barking deer). Other major constituents were magnesium (3% in antlers of chital, hog deer and barking deer, 2% in sambar and swamp deer), sodium (4% in antlers of hog deer, 2% in chital, sambar and swamp deer and 1% in barking deer), iron (2% in antler of sambar and 1% in antlers of all other deer) and potassium (1% in barking deer). Rests of the elements were below 1% level (Fig. 4.24). Although phosphorous is a major constituent however it could not be analyzed. Two elements viz. uranium (U) and cadmium (Cd) were not detected in any antler samples. Table 4.9 indicates variation in the mean concentration of elements in antlers of different deer species but, the range of concentrations of elements were overlapping in most of the antlers of deer species. The amount of potassium was remarkably high in barking deer antler. The concentration of barium was higher in swamp deer. The concentration of iron and nickel were higher in sambar antler as compared to other deer species. Concentration of copper was lower in antler of chital compared to other antlers. Though these few differences can be used in characterizing

antlers of various species more samples may be used to validate it. The Discriminant Function Analysis of mean elemental concentration could cluster antlers distinctly.

In the present study Ba concentration were found 33.03 ppm, 128.57 ppm, 207.55 ppm, 55.42 ppm and 143.86 ppm in chital, sambar, swamp deer, hog deer and barking deer respectively, a high level as recorded by Prudey (2004) from chronic wasting disease (CWD) cluster zones in North America (181 ppm) in respect of the reference levels of 5 ppm in bone matrix (Anon. 1980; Pais and Benton 1997). Analysis of plants and soil will be needed to check the level of barium in surrounding. Strontium level was also relatively higher in antler of all species chital (90.18 ppm), sambar (64.79 ppm), swamp deer (71.79 ppm), hog deer (66.34 ppm), and barking deer (289.71 ppm) in comparison to the reference. The barium and strontium levels found in this study were well below the concentrations of these two elements analyzed by Purdey (2004). Since an insufficient number of studies have been conducted on the levels of these metals in antlers (Bubenik 1971; Hyvarinen *et al.* 1977), the mean reference levels of Ba/Sr in bone material have been used by Purdey (2004) as the best alternative for providing mean reference ranges of Ba/Sr in antlers.

Many micro-satellite and DNA markers are available for cervids (Anon. 1999). Extraction of DNA directly from antler has not been tried much as the rate of success in getting out DNA from such samples is low and suffers with PCR inhibitors. Guha and Kashyap (2005) found that the designed heminested PCR assays based on unique species-specific polymorphism at mitochondrial 16s rRNA gene for identification of seven species are highly specific, simple and sensitive technique. Sensitivity of the designed assays compare to normal PCR, different DNA concentrations, species cross reactivity, specificity, and stability under various physical and chemical environment clearly reveal the applicability of the assays to less-than-optimal and highly degraded samples. Two consecutive PCRs and agarose gel visualization make the assays rapid as well as easy to interpret. Because of highly species-specific primers, the designed heminested PCR assays provide reliable evidence for wildlife enforcement. These heminested PCR assays based on species-

specific polymorphism in mitochondrial 16s rRNA gene can also be adopted for the identification of a wide range of wildlife species.

In present study success was achieved in extraction of DNA from outer and core region of antlers and it is possible to extract DNA from other regions too but this needs standardization. Amplification was successful with cytochrome b (186 bp) and better amplification is possible with trial but again more standardization is required. Sequencing of PCR products from antler of different deer species can be used for differentiating one from others with the help of alignment with the available sequences from National Center for Biological Information (NCBI) on genetic data bank. Polymorphism was found and primer was developed to be used for low fragment size. Restriction enzymes specific to different deer species can be useful to differentiate them.

For identification of antler, morphometry is best when samples are intact but, for other forms of samples scanning electron microscopy, thermo gravimetric analysis and DNA can be used successfully. X-ray diffraction, X-ray fluorescence and inductively coupled plasma-mass spectrometry need more interpretation and increased sample size. Although, presence and absence of peaks in XRD observed differences in elemental intensity and concentration through XRF and ICP-MS may be used as species specific signatures for antler of chital, sambar, swamp deer, hog deer and barking deer. Therefore, the hypothesis that morphological, crystallographic (XRD & XRF) and DNA characteristics of antler of chital, sambar and swamp deer are species specific may be accepted. In addition to these few more techniques like scanning electron microscopy, inductively coupled plasma- mass spectroscopy and thermo gravimetric analysis were helpful in getting species specific characteristics. Other than antlers of these three species, antlers of two more species were analyzed for comparison purposes.

Table 4.19. Ranking order of different techniques for identifying species from antlers of different deer species.

Types of samples	Ranking for use of different techniques						
	Morphometry	SEM	XRD	TGA	XRF	ICP-MS	DNA
Complete	I	V	VI	II	VII	III	IV
Pieces	-	IV	V	I	VI	II	III
Powder	-	-	IV	I	V	II	III

Ranking order is in decreasing order: I = Best

4.7. References

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Using morphometric and analytical techniques to characterize elephant ivory

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Abstract

There is a need to characterize Asian elephant ivory and compare with African ivory for controlling illegal trade and implementation of national and international laws. In this paper, we characterize ivory of Asian and African elephants using Schreger angle measurements, elemental analysis {X-ray fluorescence (XRF), inductively coupled plasma-atomic emission spectroscopy (ICP-AES), and inductively coupled plasma-mass spectroscopy (ICP-MS)} and isotopic analysis.

We recorded Schreger angle characteristics of elephant ivory at three different zones in ivory samples of African ($n = 12$) and Asian ($n = 28$) elephants. The Schreger angle ranged from 32° to 145° and 30° to 153° in Asian and African ivory, respectively.

Elemental analysis (for Asian and African ivory) by XRF, ICP-AES and ICP-MS provided preliminary data. We attempted to ascertain source of origin of Asian elephant ivory similarly as in African ivory based on isotopes of carbon, nitrogen and strontium. We determined isotopic ratios of carbon ($n = 31$) and nitrogen ($n = 31$) corresponding to diet and rainfall, respectively. Reference ivory samples from five areas within India were analyzed using collagen and powder sample and the latter was found more suitable for forensic analysis. During our preliminary analysis, the range of $\delta^{13}C$ values ($-13.6 \pm 0.15‰$ and $-25.6 \pm 0.15‰$) and $\delta^{15}N$ values ($10.2 \pm 0.15‰$ and $3.5 \pm 0.15‰$) were noted.
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Keywords: Elephant ivory; Schreger angle; Morphometry; Inductively coupled plasma analysis–MS; Isotopic analysis; X-ray fluorescence

1. Introduction

India being one of the 12 identified mega-biodiversity nations has 8% of the world's biodiversity with 60% of the world's tigers, 50% of Asian elephants, 70% of Asian rhinos and harbours the only population of Asiatic lion in the wild [1]. Illegal trade in wildlife and its products is a major threat and concern for conservation of endangered species throughout the world. Major illegal wildlife trade exists in skin, ivory, horn, antler, bone, live animals, feathers, nails, claws and pod. The illegal wildlife trade has been estimated to be worth US$ 5 billion which in economic terms ranked second after the drugs [1].

Over the years, poaching of megavertebrate species has depleted their numbers. In India, 75 mammal species out of 129 mammals listed in various Schedule categories under Wildlife (Protection) Act 1972 are under threat from illegal trade, of which 25 mammal species are included under the endangered categories of Schedule I and II.

Ivory being one of the highly priced article is illegally traded and the estimated annual world demand for ivory during the 1980s was 500–700 tonnes [2]. The African elephant (*Loxodonta africana*) was initially placed in Appendix II of CITES and its ivory was permitted for trade in the global market this was justified because of its large population and the considerable volumes of ivory generated from the presence of tusks in both males and females. However, was the Asian elephant (*Elephas maximus*) was listed in Appendix I of CITES and in Schedule I of the *Indian Wildlife (Protection) Act* 1972

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due to its endangered status. In spite of such legal protection, ivory from Asian elephant has always been found in the illegal trade. This is a major cause of concern for the conservation of the Asian elephant as large number of elephants were being poached every year [3].

The ban on trade in African elephant ivory in 1989 by CITES was a reaction to the significant decline in its populations from poaching [3]. However, the ban was relaxed for three African countries namely Botswana, Namibia and Zimbabwe in 1997 to permit a one-time sale of 60 tons of ivory [4]. The continued poaching of elephants in India [3] is a clear indication that illegal ivory trade is still in existence from Asian sources. This is probably because Asian ivory is more valuable than African [3]. Due to the demand for Asian ivory, widespread poaching has skewed the sex ratio in several populations [5]. The adult male to female ratio of the Nilgiri elephant population in India shifted from 1:5 in 1981 to 1:15 in 1998 [5] and further to about 1:25 by 2005 [6].

Ivory of African and Asian elephants is indistinguishable, particularly in the processed form, and thus it is almost impossible to trace the origin of tusks [7]. Enforcement of wildlife protection laws is often hampered by lack of proper methods to identify the species as well as source of the ivory [8]. Therefore, the present study aims to characterizing elephant ivory using different techniques to help enforcement agencies in getting rapid and reliable identification of seized materials and presentation of evidence in courts on the origin of such material.

Morphometry has been used extensively to differentiate species. Schreger angle of ivory is one of the important morphometric characters to be used for differentiation [9]. Elemental analysis could potentially help in distinguishing Asian from African ivory. Isotopic analyses is also useful in determining the source of origin and hence, has important role in forensics [9]. Isotopes have been successfully used to determine the source of African ivory [10]. For instance, carbon, nitrogen and strontium isotopes provide information on feeding habits, water stress and geology, respectively.

2. Materials and methods

Ivory reference samples of Asian and African elephants were used for analysis by different morphological and analytical techniques (Table 1).

Table 1
Different techniques used in characterizing Asian and African ivory

Techniques applied	Asian ivory	African ivory
Schreger angle	28	12
X-ray fluorescence (XRF)	5	5
Inductively coupled plasma-AES	5	3
Inductively coupled plasma-MS	3	3
Isotopic study	31	–

3. Morphometric technique

The Schreger angle pattern is a characteristic structural feature of the dentine portion of elephant tusk (Fig. 1). These are sets of intersecting lines radiating in spiral fashion from the axis of the tusk [9]. The angles are formed when dentinal tubules, produced by odontoblasts move towards the tusk axis during dentine deposition [11]. The Schreger angle are either centripetal or centrifugal. The Schreger angle technique has been used by in USA and by CITES to distinguish tusks of African ivory and mammoth to prevent illegal trade. Schreger angle measurements have widely been used to distinguish ivory of different species [9,12,13].

Schreger angles were examined on polished transverse section of tusks. For getting the best Schreger angle photographs, xeroxing and scanning of sample were tried. Scanning gave the best visibility of angles. A 10–20 angles were measured manually or by using software [14], at three different regions (central, middle and periphery) of Asian elephant ivory ($n = 28$) and African elephant ivory ($n = 12$).

4. Analytical techniques

4.1. X-ray fluorescence

X-ray fluorescence is an non destructive technique for elemental analysis. The X-ray spectrum reveals a number of characteristic peaks. The energy of the peaks leads to identification of the elements present in the sample (qualitative analysis) and intensity provides the relevant elemental concentration.

Five Asian and five African ivories were used for this analysis. Cross-section of ivory samples were cut and polished. Small pieces of 35–40 mm diameter and less than 10 mm thickness were directly used for analysis. Samples were desiccated overnight and analyzed under X-ray spectrometer (Siemens SRS 3000). X-ray fluorescence technique is a dry technique which gives intensity of various elements present.

4.2. Inductively coupled plasma-atomic emission spectrometry (ICP-AES)

ICP-AES is a powerful analytical tool for determinative elemental analysis. Detection limit is around 1 ppm. Three to five samples of Asian and African ivory were analyzed. Powder samples were digested by six treatments of 15 ml mixture of hydrofluoric acid (HF) and perchloric acid ($HClO_4$) each. Followed by two treatments of Perchloric acid and after adding 15 ml 10% HCl to the dried sample it was heated. This solution was made-up to 100 ml by adding distilled water. Prepared solution was analyzed through ICP-AES (Jobin Yvon JY 70 plus) spectrometer. Instrumental concentrations of various elements were transformed into parts per million using standard formula.

4.3. Inductively coupled plasma mass spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry is one of the advanced techniques to know the elemental details of the

samples analyzed as its detection level is very low, typically in parts per billion and parts per trillion. Teflon crucibles were cleaned and labelled for various samples. Approximately 0.1 g of powder sample was measured and 10–15 ml of mixture of concentrated nitric acid and hydrofluoric acid in 1:2 ratio was added. These sample was boiled with the lid on the crucible, until the sample dried. Again the same mixture was added and the sample was boiled further without the lid on the crucible. 5 ml of perchloric acid was added to these samples and boiled without lid. Same step was followed and completely dried samples were extracted with 20% HCl. The sample solution was made-up to 100 ml by adding distilled water and kept ready in plastic bottles for instrumental analysis using Perkin-Elmer SCIEX ICP-Mass Spectrometer model ELAN DRC-e.

4.4. Isotopic analysis

Isotopic ratio mass spectrometer (CE Instruments Flash EA, 112 series, ThermoQuest) was used. Standard used for the analysis were Pee Dee Belemnite for carbon analysis and atmospheric nitrogen for nitrogen analysis. Tusk samples from five states, Uttaranchal, North East, Tamil Nadu, Karnataka and Kerala were collected. Collagen was extracted from powdered ivory samples [15]. We also measured isotopic ratio using powdered samples directly. Initially collagen as well as powder from four ivory samples were analyzed for carbon isotopes in isotopic ratio mass spectrometer. The carbon isotopic values of both of these (collagen and powder form) were highly correlated and thus, we analyzed only the powder form of the samples as this was quicker. In total 31 samples were analyzed for carbon and nitrogen isotopes.

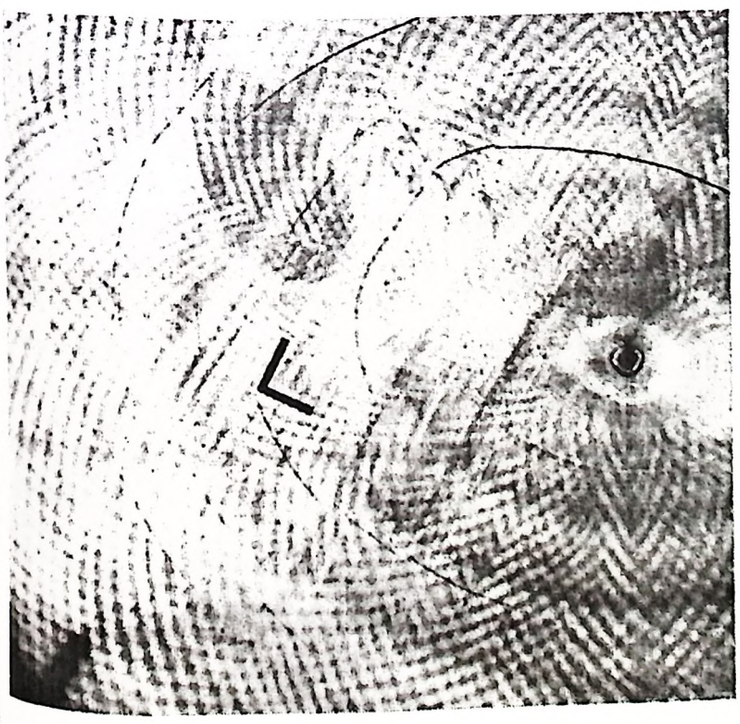

Fig. 1. Tusk dentine portion showing concave Schreger angles.

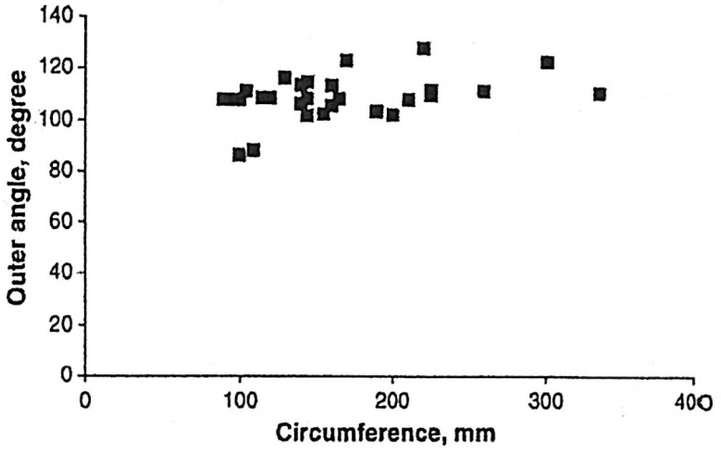

Fig. 2. Relationship of elephant tusk circumference with outer Schreger angle.

5. Results

5.1. Schreger angle

Circumference of tusk (an indicator of age of the animal) and the value of Schreger angle were plotted to know the relationship between them in case there was a change in the angle with age. There was no relationship between circumference of elephant tusk and Schreger angle values measured at the outer portion of the tusk (Fig. 2).

When all angle values of various ivory samples from three different zones namely central, middle and outer were plotted in 3-D graph, the mean value of outer Schreger angle for African ivory above are 120° and for Asian ivory these are below 120° (Fig. 3). Fig. 4 indicates mean of Schreger angle values taken at all the three portions in Asian (91.1 ± 0.70) and African (103.6 ± 1.35) ivory.

Variation in Schreger angle values at various zone of African and Asian ivory indicates that outer angle value of African ivory was higher (>120°) as compared to Asian ivory (<120°) (Fig. 5). But the middle and inner angle mean values were found to overlap in the two species.

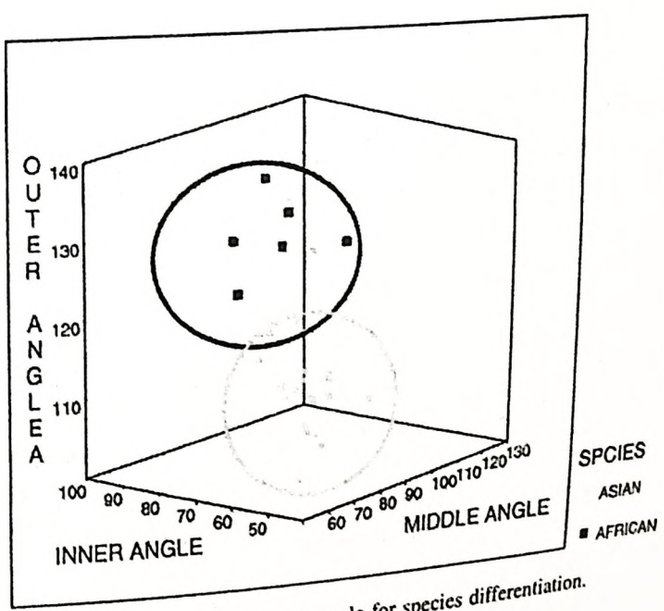

Fig. 3. Use of Schreger angle for species differentiation.

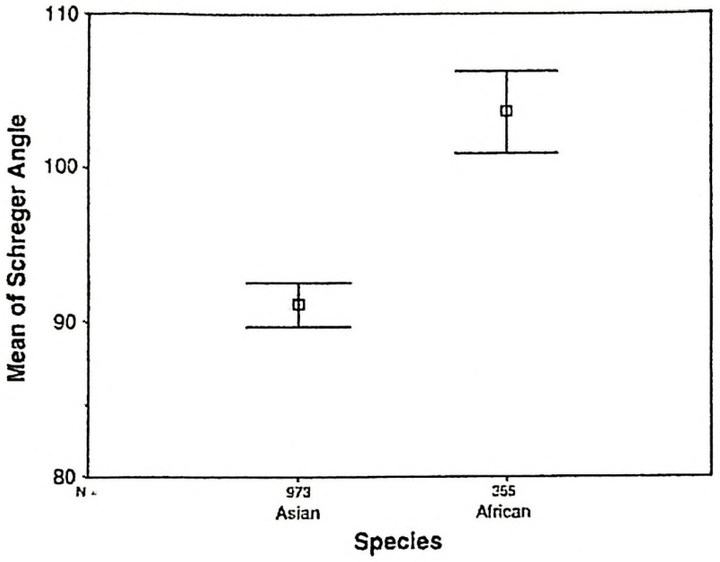

Fig. 4. Mean of Schreger angle (from all three portions of tusk) Asian and African ivory.

5.2. X-ray fluorescence

Intensities of elements such as Fe, Ni, Cu, Zn, Nd, Hf, Os, Cr, Ce, Dy, Ca, K, Cl, S, Sr, P, Cs, Ti, Mg, Al, Si and Na were obtained using X-ray fluorescence techniques. Fig. 6 reveals that the intensity of strontium in Asian ivory ranges from 1.24 to 1.5 and in African ivory from 1.8 to 2.04 kcps. Intensity of hafnium in Asian ivory ranges from 0.24 to 0.27 and 0.38 to 0.43 kcps in African ivory. Strontium and hafnium intensity were thus higher in African ivory than in Asian ivory. However, there were only marginal differences in intensity of phosphorous, calcium and halogen.

5.3. Inductively coupled plasma-atomic emission spectrometry

Mean elemental concentrations (ppm) in Asian ($n = 5$) and African ivory ($n = 3$) indicate that phosphorous, magnesium and chromium have higher concentrations in Asian elephant ivory as compared to the African elephant ivory, whereas concentrations of silicon, calcium, strontium, barium, iron, manganese and zinc were found to be higher in African ivory as compared to Asian ivory (Table 2 and Fig. 7). Concentrations of barium, strontium, silicon, iron, manganese, zinc, phosphorous and magnesium were relatively consistent in all samples of the Asian ivory. On the other hand, only barium concentration was found to be consistent in African ivory.

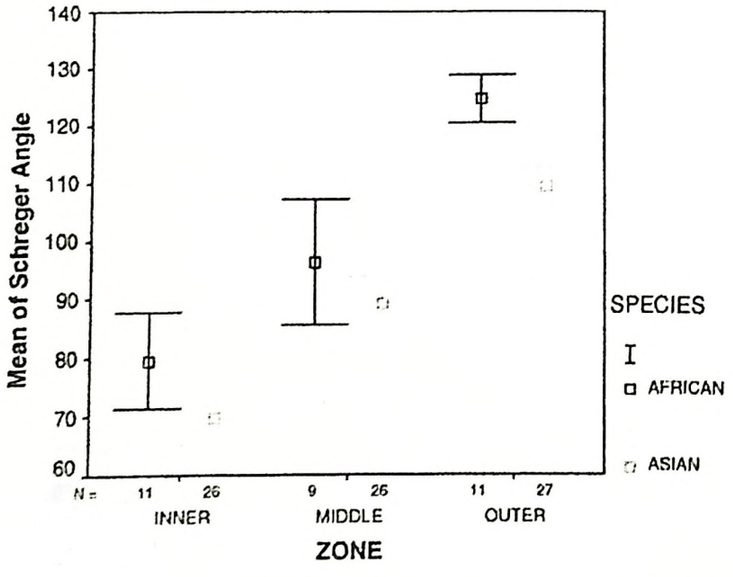

Fig. 5. Variation in Schreger angle at various zone of African and Asian ivory.

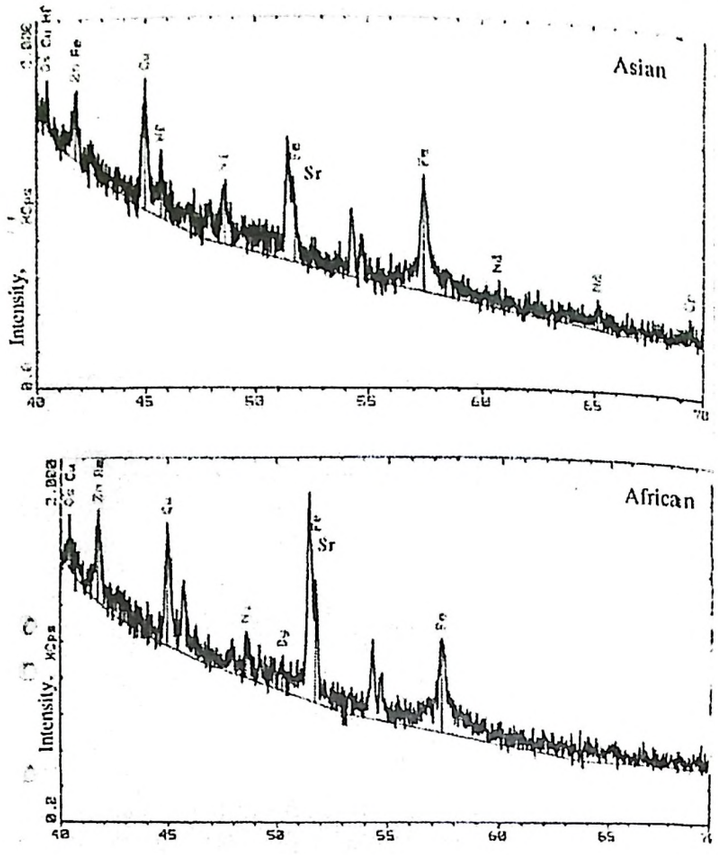

Fig. 6. XRF spectrum output of Asian and African ivory.

5.4. Inductively coupled plasma mass spectrometry

Analysis through ICP-MS reveals that the concentration of vanadium (V), samarium (Sm), europium (Eu), gadolinium

Table 2
Mean elemental concentration of Asian ($n = 5$) and African ivory ($n = 3$) calculated through ICP-AES in parts per million

Elements	Asian	African
P	89536.2 ± 3238.29	83364.2 ± 6214.68
Si	72.64 ± 2.64	117.76 ± 1.93
Ca	158585.5 ± 3443.55	177840.3 ± 48053.2
Sr	113.3 ± 19.81	221.3 ± 85.78
Ba	34.1 ± 9.47	56.9 ± 2.67
Mg	16797.1 ± 2278.26	12901.9 ± 9122.66
Fe	3306.7 ± 864.66	4927.81 ± 2379.99
Mn	16.9 ± 5.86	27.93 ± 20.93
Zn	35.9 ± 2.07	54 ± 28.49
Cr	23.7 ± 13.68	21.7 ± 14.23

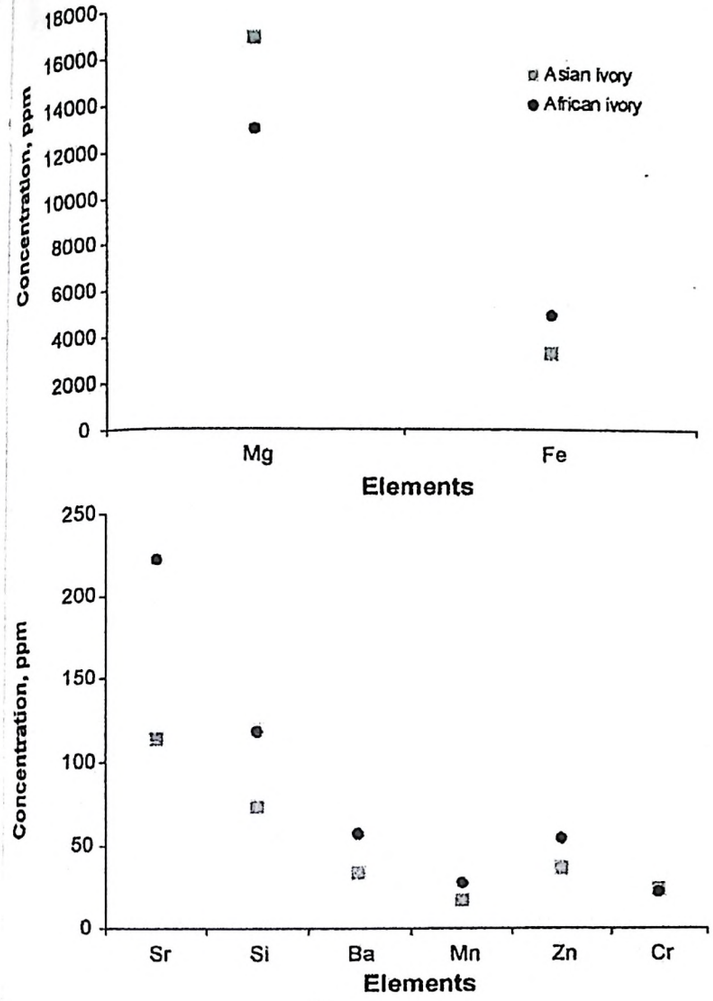

Fig. 7. Mean concentration of Fe, Mg, Sr, Si, Ba, Mn, Zn and Cr determined by ICP-AES in Asian and African ivory.

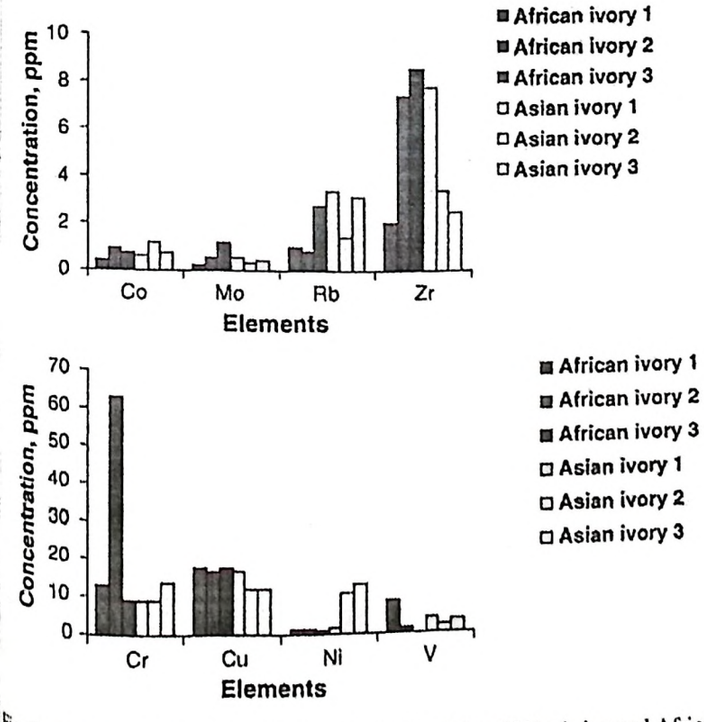

Fig. 8. Concentration of Co, Mo, Rb, Zr, Cr, Cu, Ni and V in Asian and African ivory by ICP-MS.

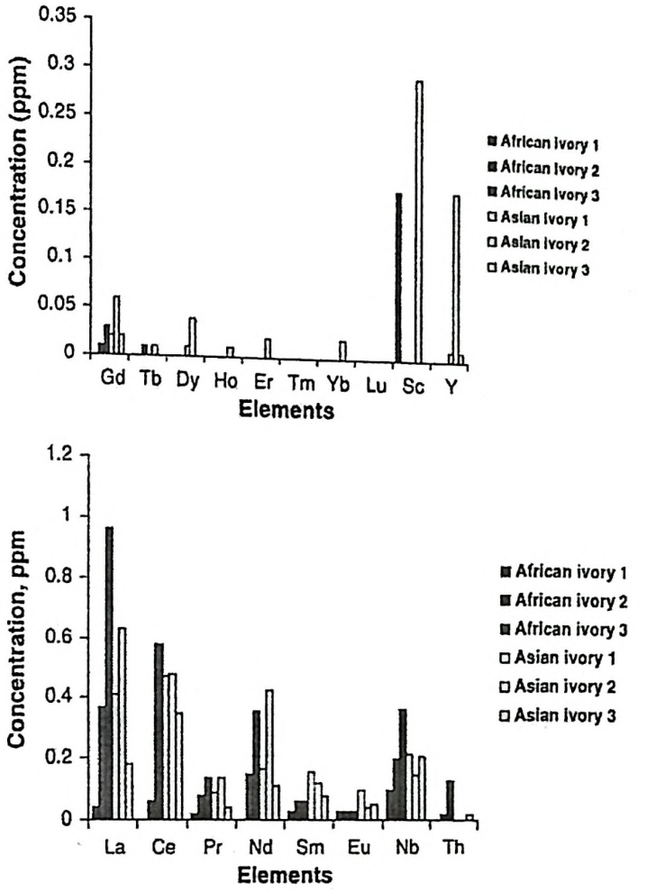

Fig. 9. Concentration of La, Ce, Pr, Nd, Sm, Eu, Nb, Th, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Sc and Y in Asian and African ivory by ICP-MS.

(Gd) and scandium (Sc) were higher in the Asian ivory whereas molybdenum (Mo) and nickel (Ni) were higher in the African ivory (Figs. 8 and 9).

Some rare earth elements, dysprosium (Dy), holomium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb) and yttrium (Y) were present only in Asian ivory and not detectable in African ivory. Yttrium was consistently found in all samples of Asian ivory (Fig. 9).

5.5. Isotopic ratio analysis

The δ^{13}carbon values of collagen and tusk powder (Fig. 10) were found to be highly correlated ($R^2 = 0.9943$). The mean δ^{13}C for African ivory (-19.4 ± 0.38 per mil) is higher than Asian ivory (-21.2 ± 0.58 per mil) with slight

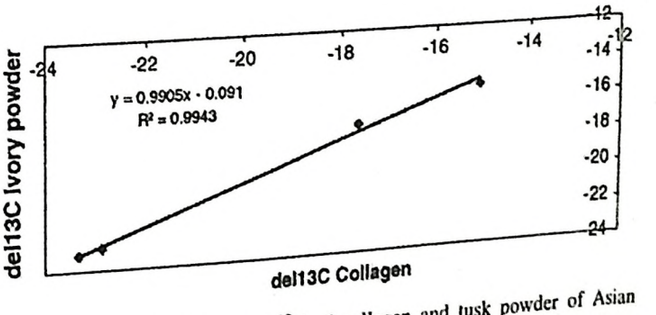

Fig. 10. Relationship between δ^{13}C of collagen and tusk powder of Asian elephant.

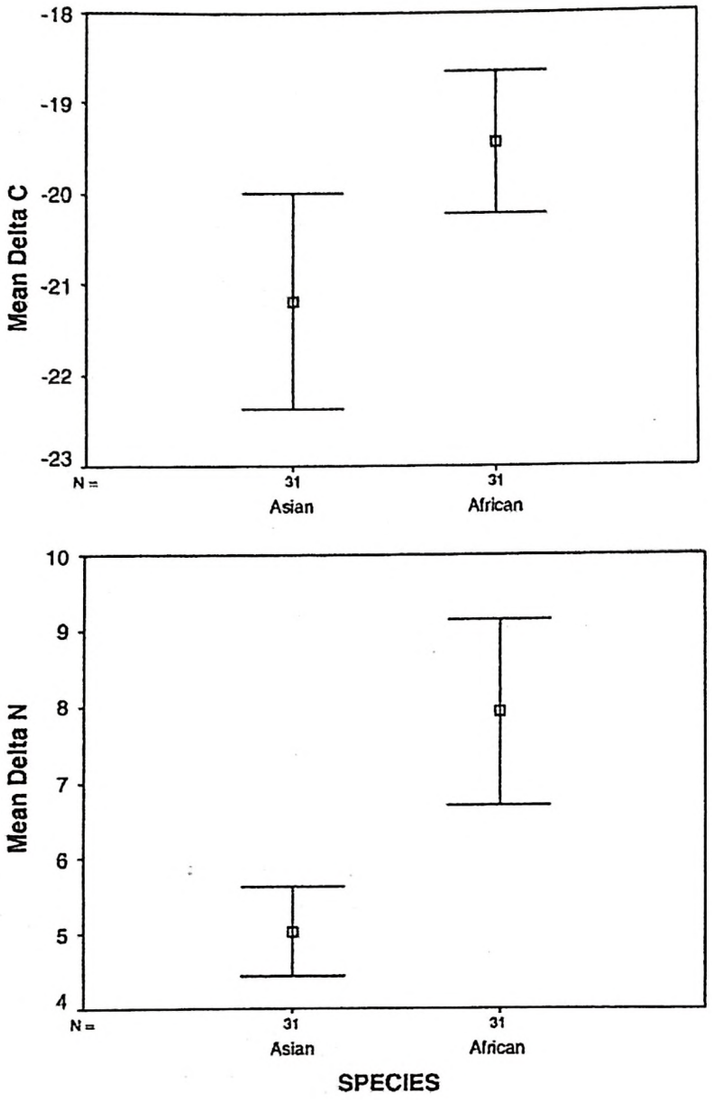

Fig. 11. Mean and standard error of carbon and nitrogen isotope of Asian and African ivory.

overlaps (Fig. 11). Mean δ^{15}N for African ivory (7.9 ± 0.59 per mil) is higher than the Asian ivory (5.03 ± 0.29 per mil) with no overlaps (Fig. 11). Values of δ^{13}C and δ^{15}N are significantly different between Asian and African elephant

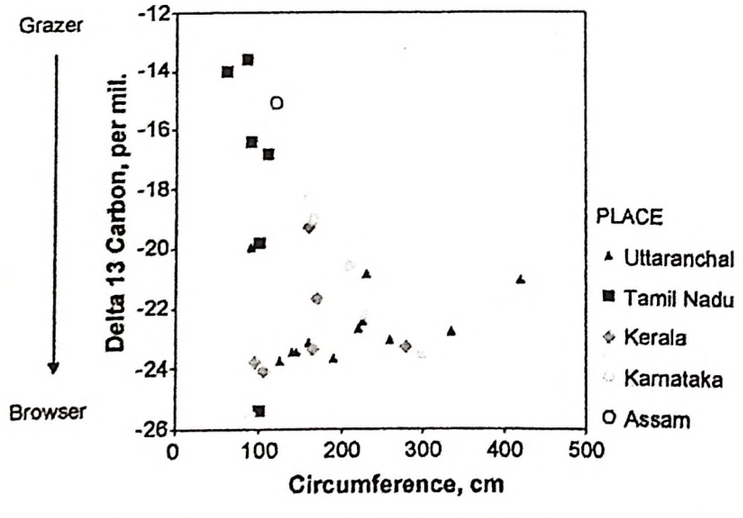

Fig. 12. Relationship between circumference of tusk and diet of Asian elephants in grass dominating habitat.

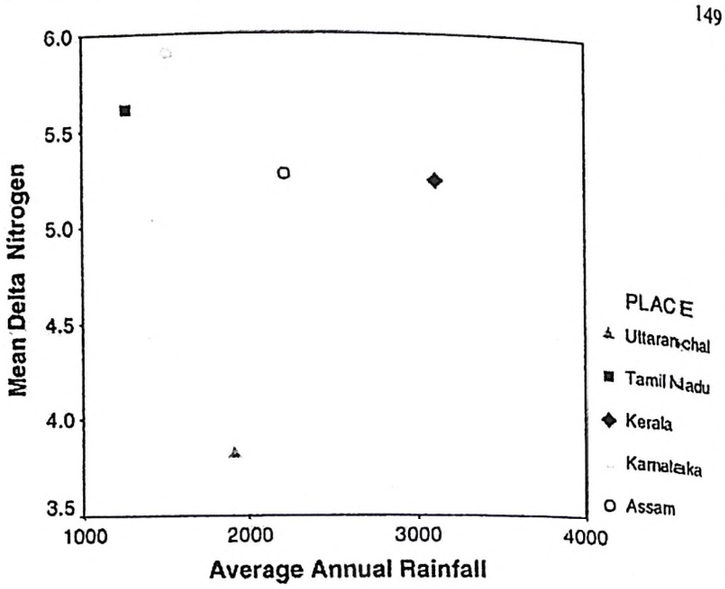

Fig. 13. Scatter plot between mean δ^{15}N and average annual rainfall.

($p < 0.001$). African ivory dataset used from van der Merwe et al. [16].

Fig. 12 reveals relationship between circumference of the tusk and diet of elephants in grass dominating habitat, where younger elephants had carbon isotopes values ranging from −18 to −13 per mil, where as adult elephants had values ranging from −20 to −26 per mil.

Scatter plot between mean value of δ^{15}N and average annual rainfall indicates that different regions forms separate cluster (Fig. 13). However, the mean values of δ^{15}N of Assam and Kerala were found overlapping.

Ivory from State of Karnataka has medium to high nitrogen isotopic ratio and the Uttaranchal has low nitrogen isotopic ratio. Whereas, ivory from Assam, Kerala and Tamil Nadu had medium nitrogen isotopic ratio (Fig. 14).

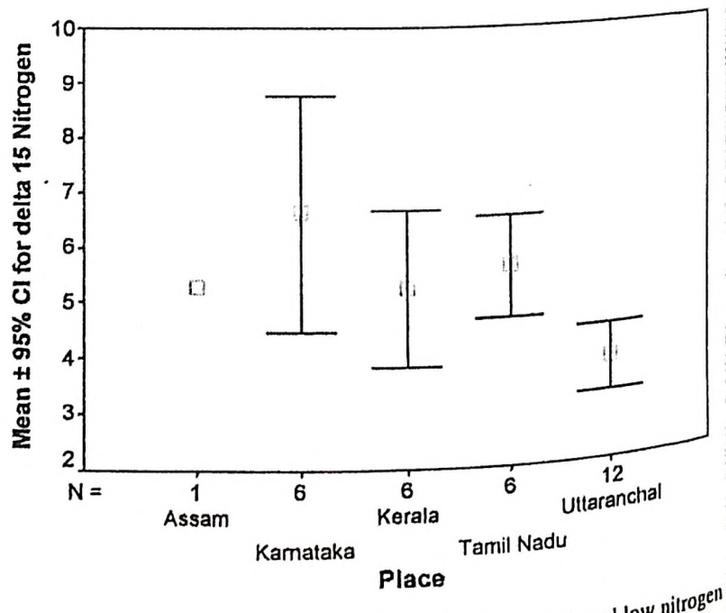

Fig. 14. Error bars showing Asian elephant tusk having high and low nitrogen isotopic ratio.

6. Discussion

6.1. Schreger angle

Identification of ivory and its substitutes is important to strengthen enforcement agencies to curb the illegal trade of ivory. Although there have been some attempts to identify African ivory from various regions [12,17] there is insufficient work on identification of Asian ivory. Espinoza and Mann [17] tried identifying ivory and its substitutes through their cross sections, and found that each ivory type is morphologically distinct [9]. Hanfee [18] in her handbook gives keys to identification of ivory of Asian and African elephants and also "fake ivory" based on visual observation. Schreger [19] in his paper is credited with the description of Huntre–Schreger bands in enamel. The presence of unique pattern of Schreger lines in transverse sections of proboscidean ivory created by the structure of microscopic dentinal tubules [20] is a distinguishing characteristic from other ivory forms. The intersection of these lines forms concave and convex angles; the concave angles open medially and the convex angles open towards the periphery. Two zones are visible of which the outer one is more distinct. Mammoth has Schreger angles less than 90° whereas extant African and Asian elephants have angle greater than 105° [9].

This study showed that mean Schreger angle value on the outer portion of ivory is more than 120° in African and less than 120° in Asian elephant (two outliers have to be rechecked). Schreger angle of mammoth was reported to be 73.21° ± 14.71 and of African ivory was 124.15° ± 13.35 [21]. We have attempted in this study to identify ivory in intact, pieces or processed forms. We observed that Schreger angle value is not dependent on age of the elephant and that Schreger angles on the outer portion are most suitable to distinguish African and Asian ivory. The ability to distinguish tusk dentin of proboscidean taxa on the basis of Schreger angle will be useful for forensic scientists, wildlife conservationist and archaeologists [12]. This is also a nondestructive forensic method for identification [9].

6.2. X-ray fluorescence

Ivory of elephants comprises chiefly of dentine which are made by the inorganic compound known as dahllite with the general formula $Ca_{10}(PO_4)_6(CO_3)H_2O$ [16]. Intensities of 22 elements were obtained using X-ray fluorescence techniques. The results show that strontium and hafnium was higher in concentration in African ivory than in Asian ivory. However, there were marginal differences in intensities of phosphorous, calcium and halogen. The concentration of strontium and hafnium could serve as species specific signatures to identify African and Asian ivory wherever morphometric techniques are not useful.

6.3. Inductively coupled plasma-atomic emission spectrometry

The analysis shows that phosphorous, magnesium and chromium has higher concentration in Asian elephant ivory as compared to African elephant ivory whereas concentration of silicon, calcium, strontium, barium, iron, manganese and zinc were higher in African ivory as compared to the Asian ivory. The concentration of barium, strontium, silicon, iron, manganese, zinc, phosphorous and magnesium were consistent in all samples of the Asian ivory. On the other hand, only barium concentration was found consistent in the African ivory. Difference in the ranges of the above elements in African and Asian ivory will be useful in distinguishing the two ivories.

6.4. Inductively coupled plasma mass spectrometry

Concentration of vanadium, samarium, europium, gadolinium and scandium are higher in the Asian ivory where as molybdenum and nickel are higher in the African ivory. Other rare earth elements dysprosium, holomium, erbium, thulium, ytterbium and yttrium are merely detectable only in Asian ivory and not detectable in African ivory. The consistence of yttrium concentration in all samples of Asian ivory and not detectability of this element in African ivory can be useful to distinguish ivories of the two continents.

6.5. Isotopic analysis

Scientist have analyzed isotopic ratios for ivory of African elephant for determining source area [16]. This technique has yet not used in Asian ivory to know their origin though, some isotopic work on bones of Asian elephants has been done [22]. The trivariate isotopic analysis namely stable carbon isotope ratio ($^{13}C/^{12}C$) in elephant bone collagen shows the mixture of C_3 foliage and C_4 grasses in the diet, and are directly proportional to the density of C_3 browse [23], nitrogen isotope ratio ($^{15}N/^{14}N$) in bone collagen is related to rainfall and water stress [24,25] and strontium isotopes ($^{87}Sr/^{86}Sr$) reveal local geology [25,26].

This study shows that isotopic ratio from tusk powder and collagen is highly correlated. Thus, forensics examination from powder samples is more suitable as sample preparation is limited and results can be obtained quickly as compared to collagen. Some distinction across regions may be possible on the basis of browser versus grazer populations; for instance elephants from Karnataka, Kerala and Uttaranchal seem to browse to a greater extent than those in Tamilnadu and Assam but this could also be due to rapid turnover of collagen and fluctuating isotopic signatures in younger animals [27]. Thus, tracing the source of ivory from carbon isotopic ratios alone is not possible.

Mean $\delta^{13}C$ for African ivory is higher than Asian ivory but there is overlap between the values from the two continents. Mean $\delta^{15}N$ for African ivory is higher than Asian ivory with no overlaps in the standard errors; this could thus serve as a tool to distinguish the species. Mean $\delta^{15}N$ and average annual rainfall shows that different states can be separated into different cluster. However mean of $\delta^{15}N$ values of Assam and Kerala are overlapping might be due to low sample size. The presence of low nitrogen isotopic ratio in Uttaranchal elephants tusk,

medium to high in Karnataka elephants tusk, and medium nitrogen isotopic ratio in Assam, Kerala and Tamil Nadu elephant tusk are due to presence of different plant type and food species in different states.

We conclude that it is possible to differentiate Asian ivory from African ivory using Schreger angle, isotopic study, XRF, ICP-AES and ICP-MS. With such database, different techniques can be applied depending on the type of seized material received such as whole tusk, piece, powder and artifact. Isotopic study will be useful for wildlife managers and law enforcement authorities to know the origin of seized material and take measures to curb poaching. Strontium isotope data will add to the robustness of these results.

Acknowledgements

We are thankful to the Director, Wildlife Institute of India for the support for research and development work. We would like to thank the Council of Scientific & Industrial Research, New Delhi and the Secretary, MoEF, New Delhi, India for funding support without which it was not possible to present our work at this forum. We are grateful to the Chief Wildlife Wardens of Uttaranchal, West Bengal, Assam, Kerala, Karnataka and Tamil Nadu for providing samples for research purpose. Our sincere thanks to the Director, Wadia Institute of Himalayan Geology (WIHG), Dehradun and their faculty and technicians for their help while using their instrumental facilities. We are grateful to Dr. M.S. Sheshshayee, Gandhi Krishi Vikas Kendra, University of Agricultural Sciences, Bangalore, for helping out with isotopic analysis. Sincere thanks to Faculty, Staff and Friends at Wildlife Institute of India. Thanks to R. Jaipal for statistical analysis. Thanks to Laboratory Assistant Sanjay Chouniyal for his sincere hard work to see that all samples were prepared. We are thankful to our colleague in the laboratory for encouragement and my family for their patience and encouragements. Last but not least thanks to all those who directly or indirectly help in this paper preparation.

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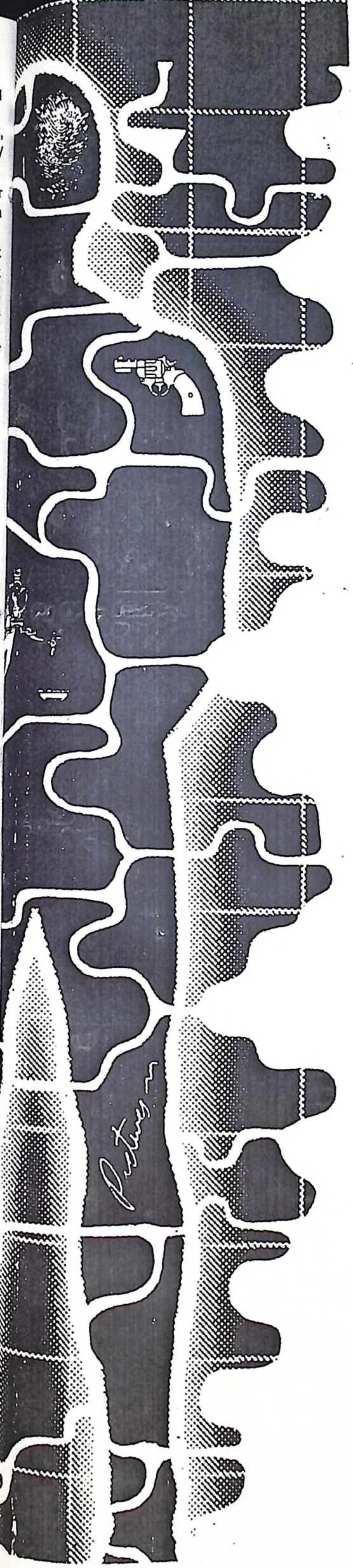

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WIL-FP-05
Identification of the Origin of Meat Samples Sold in Markets

Adrian Linacre, Forensic Science Unit, University of Strathclyde, UK; Hsing-Mei Hsieh, Department of Forensic Science, Central Police University, Taiwan; Chin-Cheng Tsai, Li-Chin Tsai, Department of Forensic Science, Central Police University, Taiwan; Nu-En Huang, Rocky Tai-Ping Shih, Criminal Investigation Bureau, Taiwan and James Chun-I Lee, Department of Forensic Science, Central Police University, Taiwan.

Aim of Paper: In this study, a DNA test was used to screen random samples of processed meat products bought from supermarkets. Process meats are likely to contain degraded DNA, therefore mitochondrial DNA (mtDNA) was considered more suitable than nuclear DNA for this analysis. The study showed a large number of meat samples were mislabeled, with cheaper quality meat being labeled as a more expensive variety. Introduction: Consumers rarely have a problem identifying fresh meat when bought at markets or in shops. The particular colour and shape of fresh beef can be distinguished from pork or poultry when the meat is fresh. Processed meat, such as sausage, jerky, and canned foods, pose more of a problem as the product can not be seen. In such cases where the meat is thought to be unrepresentative of the advertised product, a dispute between the consumer and the seller may arise. This is particularly the case if the customer feels that they have been sold inferior quality meat. The cytochrome b gene on the mitochondria has been used successfully in species identification and in taxonomic and phylogenetic studies. The full cytochrome b gene sequence is over 1.1 kb in size and it is therefore a lengthy process to compare data. A partial sequence of the cytochrome b gene, which is 402bp in size, was evaluated and proved to be suitable for animal identification in a recent study. The reason for using the cytochrome b gene is that it has many advantages over other loci; it is encoded on the mitochondrial genome and therefore more abundant than nuclear DNA, and also less susceptible to degradation. It can easily be amplified from poor quality samples and the whole PCR locus directly sequenced. In this study this DNA locus was used to identify the species from which different meats originate from a range of meat samples collected from markets in Taiwan.

Materials and methods: A total of 87 meat samples were collected from different markets in Taiwan. DNA was extracted and amplified using the cytochrome b primers (L14724 and H15149). The resulting PCR products were directly sequenced. The resulting sequence was compared to the EMBL database for alignment to major meat-producing species.

Results and Discussion: Amplification of the cytochrome b gene produced a 402 bp product for all the samples. All of the sequences were compared with the sequences registered in GenBank and EMBL by GCG computer package. Of the 87 samples, there were 11 that did not conform to the original specified material. The ratio of inconsistent samples to consistent samples was therefore approximately 12.6 %. All the inconsistent samples were advertised as being beef but were found to be more closely related to pork. The DNA sequence similarities of the other consistent samples were all higher than 98 %, and the sequence diversity (variations per 100 bases) was less than 3 %. Previous studies have shown that the percentage range of sequence diversity in the same species for this part of the cytochrome B gene was from 0.25% to 2.74 % (less than 3 %), and that between the different species was from 5.97% to 34.83%. The genetic distances between intraspecies or inter-species were generated by Kimura's 2-parameter model. The genetic distances between intraspecies, ranged from 0.00 to 1.77 (pig), and 0.00 to 2.29 (cow). The genetic distances between interspecies (pig and cow) ranged 19.11 to 21.90. The value represents the evolutionary distance between two sequences. Our previous study showed that the genetic distance between the different species ranges from 6.33 to 40.59.

Conclusion: We have illustrated the value of a simple DNA test to identify the animal species used in processed meats. If such a test is used frequently, the confidence of consumers in the identity of the processed meat they buy and consume should increase.

Keywords: Meat, DNA, Cytochrome B

WIL-FP-06
Application of X-Ray Diffraction to Characterize Ivory, Antler and Rhino Horn: Implications for Wildlife Forensics

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Demand in the international market and the usefulness of ivory, antler and rhino horn have led to large scale poaching of animals providing these materials. Items of these materials are used for various applications. Ivory is used for decorative show pieces because of the ornamental value. Antler is used for buttons, pistol butts, key rings and hangers. Antler and Rhino horn are both used in traditional medicine. Rhino horn is also used for dagger handles in Yemen.

In order to protect animals related to these products, laws have been framed under CITES and Indian Wildlife (Protection) Act, 1972. Two of the animal species, viz. Asian Elephant (Elephas maxima) and Rhinoceros (Rinoceros unicornis), have been placed in Appendix I of the CITES and in Schedule - I of the Indian Wildlife (Protection) Act, 1972, indicating their precarious status.

Wildlife items are illegally traded as both finished and semi-finished products. They can also appear in powdered form which makes the task of identifying species very difficult. Since the dentine and bony materials of animals are made up of crystalline apatite mineral (Calcium hydroxide ortho-phosphate; $Ca_5(PO_4)_3OH$), this can in principle be characterized by its own characteristic X-ray diffraction pattern. An attempt has, therefore, been made to characterize the materials using the X-ray diffraction technique. In this paper we discuss our preliminary results using a limited number of Ivory samples (n=4), Rhino horn (n=2), Fake Rhino (n=1), Buffalo horn (n=1) and Antler (n=3). X-ray diffraction analysis was performed using a Phillips PW 1710 X-ray diffractometer.

Sampling is an important feature of an experiment and care was taken to use a proper sampling procedure. All samples were ground for 15 minutes, resulting in crystallite particle sizes ranging from 200 –300 mesh. For the XRD analysis we used a 55-mA current and a 40 kV voltage as applied to the X-ray tube. Some of the parameters were kept constant for all samples such as: scanning range (2θ) = 25 to 55°, step size = 0.02° (2θ), and Time per step = 10 sec. These settings are based on a pre-optimisation to obtain an improved diffractogram. Most important factor for refinement was the time per step (varied from 1 to 4 to 10 sec).

Except for Rhino and Buffalo horn, the X-ray diffraction patterns of all samples of ivory, antler and bone match well with the pattern of hydroxy-apatite (ASTM file no. 9; card no. 432). This apatite mineral is hexagonal in shape. It can be rod like, needle like or even plate like, depending on the length of the atomic lattices 'a' and 'c'.

Invariably, the peaks show a broad base and there is a large background hump. The background hump is attributed to the presence of amorphous organic and non-crystalline phosphate constituents, whereas, the broadening of the peak is due to the smaller crystallite size of the apatite minerals and strain effects. On the other hand, the Rhino horn that is made up of dead protein (Keratin protein), does not show any crystallinity. No distinct peak can be observed, only numerous small peaks. It is thus possible to distinguish the Rhino horn from animal bones, ivory and antler. Upon comparison of X-ray diffraction patterns for Rhino and buffalo horn, the latter was devoid of any peak. This gives an indication for further distinguishing Rhino from other horn materials.

Diffraction patterns of ivory and antler were similar in general appearance. However, minute but distinct and consistent differences in the diffraction patterns of ivory and antler have also been ob-

served. The ubiquitous presence of a shoulder peak at d= 1.9, at 47^0 (corresponding to the 312 hkl lattice plane) is observed for antler material but is absent in ivory. The twin peak of 211 and 112 in ivory samples are well-separated giving rise to a plateau, however, the same is indistinguishable in case of antler. The 210 peaks are more prominent in antler samples than in ivory. The background line between the peaks 213 and 004/411 is concave upward in case of antler, whereas, for ivory it is like a descending slope.

In addition to this, there exist differences in terms of other parameters like crystallinity, crystallite size, state of strain and cell volume parameters that may be useful in distinguishing antler, ivory and bone materials of different species. A fake Rhino horn was also tested and it was found that it matches with the hydroxy-apatite pattern. The value of the calculated crystallite size was smaller for ivory as compared to antler. Upon plotting the angle versus relative intensity, the highest peak could be used to differentiate between all hydroxy apatite and non-hydroxy apatite minerals. Scatter plot between maximum and minimum background intensity could differentiate among ivory, rhino horn and antler. Cell parameter and cell volume of ivory and antler were useful for distinguishing these two items. The 'a' cell parameter of ivory ranged from 9.45-9.53 whereas for antler it ranged from 11.62-11.85. Crystallite cell volumes of ivory were smaller in comparison to antler.

Thus, by using this technique, which is non-destructive and requires small sample amounts, it is possible to differentiate between wildlife trade items and lead for proper implementation of Indian wildlife (protection) Act and CITES.

Keywords: Horn characterization, X-Ray Diffraction, Wildlife protection

WIL-FP-07
The Wildlife Forensics DNA Facility: A new Initiative of Wildlife Institute of India

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Mandates of the Wildlife Forensics Cell of Wildlife Institute of India are to standardize techniques for identification of species from various parts and products of different species and provide support to enforcement agencies for implementation of the *Indian Wildlife (Protection)* act 1972. As part of a five year WII-USFWS project a major emphasis has been on identification of species based on morphometry techniques and four manuals are in the process of finalization viz. "Identification of species from hair", "Characterization of species from canines", "Identification of species from claws and beaks" and "Tibetan antelope - Trade and Wildlife forensic techniques for identifying Shahtoosh hair".

One of the major problems for dealing with tissue samples sent to us, was that most of the samples were not properly preserved and it was not possible to use normal electrophoretic techniques such as IEF. Therefore, a manual was prepared on protocols for tissue sample preservation, viz. "A field guide for collecting tissue samples for Wildlife Forensics analysis." Dealing with pending tissue cases (n= 129), it has been decided to establish a Wildlife Forensics DNA facility to standardize techniques for identifying species from various animal parts. Pending cases are for tissue, processed skins, claws, canines, nails, whiskers, bones, bear bile's, musk pod, blood, meat, hair, blood stains and meat preserved in formalin, salt solution etc. We shall address the problem of standardizing protocols for extracting DNA from Wildlife Forensics materials that are highly degraded and developing markers using molecular techniques such as RAPD, PCR-RFLP, AP-PCR, Southern blotting and specific probes.

We also intend to identify the source of origin of tiger, leopard and elephant from parts and products. Four protocols were tested by us for 30 meat samples. 10% of these samples contained very good quality DNA that was used for RAPD amplification. 70% of the samples contained degraded DNA and 20% of the samples yielded very poor quality DNA that needed to be extracted again by modifying protocols. We also tested DNA protocols for other biological samples such as skin (n = 6), hairs (n = 10), bear bile (n = 4), musk pod (n = 4), antler (n = 1), ivory (n = 2) and blood (n = 5). As DNA molecules are highly stable under extreme conditions and exhibit high polymorphism, they can play a major role as molecular markers for rapid detection of Wildlife species and their products as well as the level of diversity among them. We intend to analyze one mitochondrial protein coding gene sequence (cytochrome b), two mitochondrial ribosomal RNA gene sequences (12s RNA and 16s RNA) and in nuclear DNA one un-translated region (UTR) of SON DNA binding protein gene sequence. Collaboration with zoos at Delhi, Kanpur, Chennai and Mysore has allowed us to procure reference tissue samples of around 75 species. Major constraints are funds and space for developing the Wildlife Forensics facility.

Keywords: Wildlife trade, DNA extraction, PCR-RFLP, RAPD

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Species Identification of Animals With Matrix-Assisted-Laser-Desorption/Ionization-Time-Of-Flight Mass Spectrometry (Maldi-Tof Ms) Using Keratin Structures (Siam). A New Method for Quality Control and Animal Protection

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Species Identification of Animals with Matrix-Assisted-Laser-Desorption/Ionization-Time-of-Flight Mass Spectrometry (MALDI-ToF MS) using Keratin Structures (SIAM). A new Method for Quality Control and Animal Protection Klaus Hollemeyer *(T), Wolfgang Altmeyer(#), and Elmar Heinzle (T) (T) Biochemical Engineering Institute, Im Stadtwald, Saarland University, D-66123 Saarbrücken, Germany, and (#) GENE-FACTS GbR, Science Park Saar, Stuhlsatzenweg 69, D-66123 Saarbrücken, Germany For the quality control of feathers, down and hair it is necessary to identify the tested material properly. This is because of the commercial value of some high priced raw material as well as for exclusive ready-to-use products and because of falsifications with cheap substitutes.

A further important aspect is the need to exclude material from endangered species from illegal trading often being incorrect declared or smuggled. So far the identification of feathers, down and hair is mainly performed with visual and microscopic methods. These are often time consuming and need the experience of experts. Even then up to 35% of all down can not be identified undoubtedly for example. Because of the biochemical and physical properties of these materials some of the classic identification methods fail like Fatty-Acid-Methyl-Ester analysis (FAME) for fat containing samples, Enzyme-Linked-Immuno-Assay (ELISA) for soluble antigen-antibody-reactions or the Polymerase-Chain-Reaction (PCR) using amplifiable DNA- or RNA- sequences. The latter method is hardly usable for tinted or chemically processed materials. Two-Dimension-Gel-Electrophoresis (2-D) followed by an amino acid sequence analysis is not economic and not for high throughput. The request for quantification of mixed samples can hardly be performed using these methods.

To overcome these drawbacks we recently developed the new SIAM method for the identification of the origin of feathers, down and hair and for the quantification of mixed samples exclusively using the almost insoluble proteins of these keratin structures. After a thiol reduction step samples of reference material are enzymatically cleaved by trypsin, a specific cleaving endoproteinase, No prior solubilisation or isolation steps for the structure proteins were