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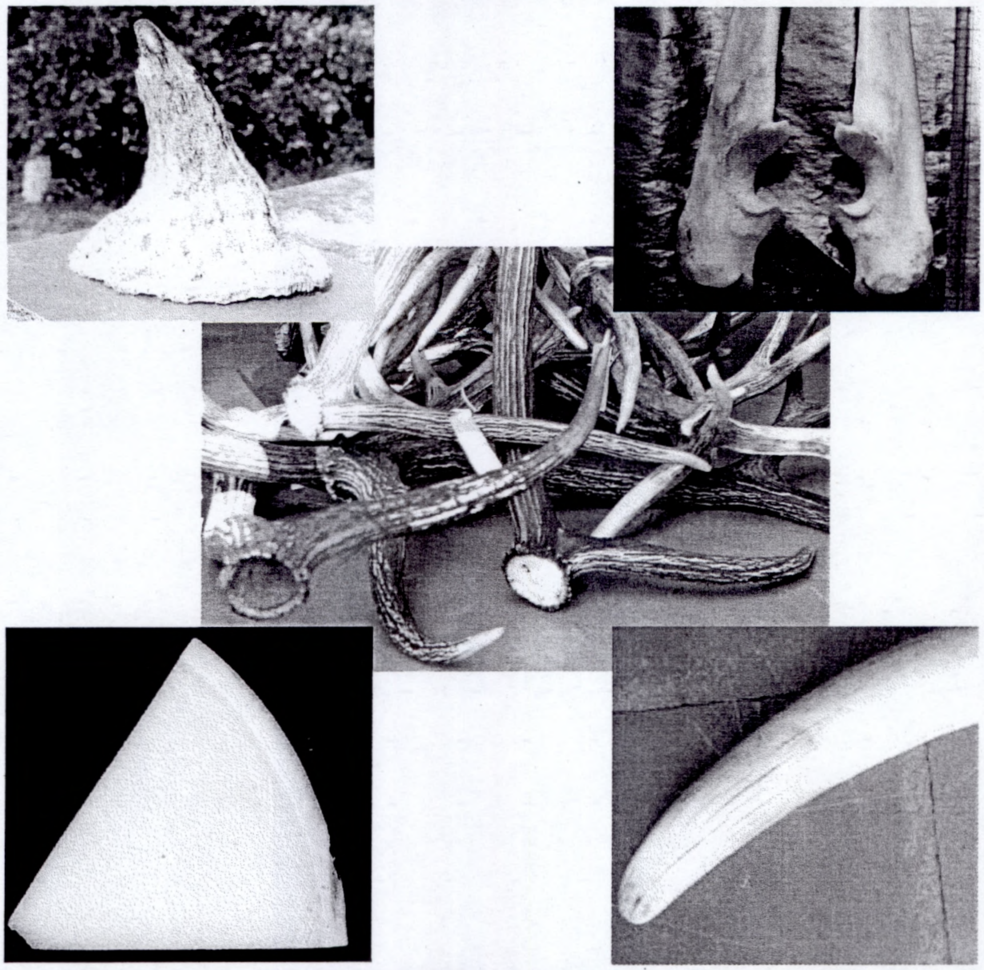
Research project titled "Characterization of species from bone, tusk, rhino horn and antler to deal wildlife offence cases"

FINAL REPORT



भारतीय वन्यजीव संस्थान
Wildlife Institute of India

2008

Research project titled "Characterization of species from bone, tusk, rhino horn and antler to deal wildlife offence cases"

FINAL REPORT

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Contents

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LIST OF FIGURES

LIST OF TABLES

Summary

Characterization of bones, tusk, 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 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*), ivory of Asian elephant (*Elephas maximus*) have also been characterized and compared with African elephant (*Loxodonta americana*) ivory, greater one horned rhinoceros (*rhinoceros unicornis)* horn and antlers of chital (*Axis axis*), sambar (*Cervus unicolor*), swamp deer (*Cervus duvauceli*). Other than these species, antlers of hog deer (*Axis porcinus*) and barking deer (*Muntiacus muntajak*) were also characterized.

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. Develop morphmetric, chemical and DNA based techniques to characterize species from bones of major animals such as tiger, leopard, lion, chital, sambar, barking deer and swamp deer.

2. Establish species specific characteristics of raw and finished products of Asian ivory and prepare protocols to differentiate from other similar products, used in the trade.
3. Investigate source-area of Asian elephant ivory.
4. Determine characteristics of rhino horn.
5. Establish species characteristics and keys to identify antler of 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 their characteristics. In this study, morphological features have been used to differentiate bones, tusk, 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 ivory". This chapter deals with introduction of elephant species, illegal trade of ivory, literature review, methods of characterization of ivory, result, discussion and references. Morphological characteristic of ivory viz., Schreger angles are very useful in differentiating ivory obtained from different species. Mean Schreger angle value on the outer portion of ivory is more than 120^0 in African and less than 120^0 in Asian elephant. At dentine portion Asian elephant ivory has different surface topography than African elephant ivory. This difference can help in differentiating Asian and African elephant ivory pieces. X-ray diffraction (XRD) of ivory indicates hydroxyapatite mineral composition. Thus, these characteristic peaks can be used for identification purpose and the specificity of the diffraction pattern can distinguish the ivory. Ivory has characteristic three exothermic peaks for ivory. Average weight loss at 1400 ^{0}C in case of African ivory is higher (48.15%) as compared to Asian ivory (47.15%). Average loss of moisture content in African ivory (3.45%) is less than Asian ivory (4.62%). Difference was observed in weight loss pattern after 1351^0 in African and Asian elephant ivory. The results show that strontium and hafnium was higher in concentration in African than in Asian elephant ivory. The concentration of strontium and hafnium could serve as species specific signatures to identify African and Asian ivory. Differences in concentration of various elements can be used in distinguishing African and Asian ivory. The consistence of yttrium concentration in all samples of Asian elephant ivory and not detectable in African elephant ivory can be useful to distinguish ivories of the two continents. This study shows that isotopic ratio from tusk powder and collagen is highly correlated. 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. It is very well possible to know the origin of tusk once all three isotopes carbon, nitrogen and strontium ratios are taken into account. The complete ranges of different techniques are useful to distinguish Asian and African ivory present in any forms.

Chapter 4 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 ^0C 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 5 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^{0}C. Weight loss pattern of antlers of different species showed remarkable differences in three temperature sections, 0-300^{0}C, 450-700^{0}C and 1000-1400^{0}C. 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^{0}C 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 at cytochrome b region. 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, ivory of different origins, Indian rhinoceros horn and antlers of different species.