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

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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

WIL-TO-01

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