Wildlife Forensics

Keywords: microsatellite, Aguila, Falco

WIL-FP-01
Genetic Variability of Raptors in Slovakia

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The aim of this work was to map the frequency distribution of chosen microsatellite DNA markers present in the wild golden eagle (Aquila chrysaetos) and Falco sp. (Falco rusticolus, Falco peregrinus, Falco cherrug) populations. Aquila and Falco sp. are included in the CITES list of endangered species. The same markers were evaluated in the group of animals living in captivity. All data presented were obtained from Slovak and Czech Republics. The DNA samples were screened using a set of polymorphic dinucleotide microsatellite markers originally cloned from Falco rusticolus. The microsatellite loci were amplified by PCR in a final volume of 20 microliters and evaluated using an automatic DNA sequencer. Allele sizes were determined by comparing them with a standard. All together, 150 individual DNA samples from both species were analyzed. The blood samples were taken mostly by the members of Slovak Environmental Inspection (SEI). Our intention was to choose those markers, which could be used as a forensic tool when illegal collection of species is suspected. The reliability of individual identification in Aquila chrysaetos is relatively low. Microsatellite databases of wild living animals as well as those kept legally, is useful for individual identification. This can be used in the struggle against nest robbery and the discrimination between individuals bread in captivity and those illegally imported from abroad. These databases can be used by the Ministry of Environment of the Slovak Republic and the Slovak Environmental Inspection.

In our work we focused our effort on finding out the relation between the occurrence of certain allels, DNA microsatellites and the geographical nesting localization, or whether there is a significant difference of some allels of certain microsatelites in different geographical regions, respectively. For the comparison we have used also individuals bred in Germany, Austria and Belgium. We did however suspect that some of these individuals originally came from Slovakia, because in the mentioned regions they do not occur naturally. For the comparison it would be probably useful to include also data from Northern and Eastern European regions, or other parts of the world. Our data show that the occurrence of certain alleles are not related to the geographical nesting location, the fact that is readable from the character of eagle nesting. On the other hand, there is a clear difference in frequency of occurrence of certain alleles in the genome of the birds bred in captivity and the wild individuals. The difference becomes clear when the frequency of occurrence of alleles studied in these two groups are compared.

An additional result of our work was the achievement of information on genetic variability, mainly of golden eagles, whose wild population in our country consists only of few dozen individuals. Because of the high frequency of inbreeding both in wild and in captivity, the variability between the individuals is reducing. Information on population variability is important for the real reflection of the health of the population and their protection.

Our DNA microsatellite databases can be utilized also by the breeders to achieve non relative breeding, which may lead in an increased genofond variability. Thus, one of the results of our study is the discovery of the very low genofond variability of wild living eagles in Slovakia. In future it would be necessary to widen our eagle population by using individuals with a heterozygotic genotype. Heterozygosity of analyzed DNA samples ranges between 0.09-0.61. In future, we would like to compare our data with similar data, obtained from other parts of Europe. Presently, our database is enlarged by other DNA microsatellites, in which significantly more polymorphism is expected, mainly in the eagle population.

WIL-FP-02
DNA Typing from Elephant Tusks and Rhinoceros Horn - Strategies and Applications in Wildlife Conservation and Animal Forensics

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Introduction: With this study, we demonstrate the potential of ancient DNA analysis as a tool for identifying the source of samples thought to be derived from threatened or endangered species. These analyses are shown to be successful even for DNA extracts from sample materials that are derived from highly processed and degraded objects. Furthermore, we introduce an approach towards the molecular identification of the African elephant and its discrimination against the Indian elephant as well as the identification of three subspecies of rhinoceros. Since the samples in question are often contaminated by human cellular material, the analysis system was designed in a way to exclude the human sequences from being co-amplified. African elephants (*Loxodonta africana*) and the three subspecies of rhinoceroses (*Ceratotherium simum, Rhinocerus unicornis* and *Diceros bicornis*) have been considered as highly endangered species for many years. Thus, trading with any products derived from these animals was made a punishable offence. However, African elephants are still poached on account of their meat and tusks. From the tusks ivory objects like e.g. statuettes are manufactured and dealt with. Similiar conditions hold true for rhinoceroses. Rhinoceroses are poached because of their horn, which is pulverized and, for "medicinal properties", mainly traded throughout East Asia where these powders are regarded as an aphrodisiac. This ongoing illegal trade is a serious threat to these wildlife species although high penalties are imposed on anyone taking part in the trade. However, in many cases problems arise in providing evidence to convict these people. This holds particularly true when only trace amounts of the suspicious material are at hand or when the respective animal products (e.g., „medicines") cannot be identified by morphological inspection. In addition, the newly installed partially legalized trade with elephant tusks raised the problem to identify and discriminate illegal from legally traded material. To address this question, reliable methods, programs and databases have to be developed to ascertain the identification of the traded material. In such cases, molecular methods can supply decisive information about the specimens and materials, independent from their outer appearance. The employment of a reliable molecular identification would enable more efficient law enforcement. The PCR (Polymerase Chain Reaction) enables us to carry out DNA analyses even on highly processed or physically transformed materials. For this purpose, it is necessary to design primer systems that amplify short PCR products, since degraded DNA often does not contain fragments exceeding about 200 bp in length. With the present study we show, that this is possible by example of species determination by amplifying and sequencing amplicons from the cytochrome b gene.

Material and methods: Our studies were performed on elephant ivory and rhinoceros horn as confiscated by the customs of the Frankfurt International Airport. Small samples of less than a gram were taken from the bottom and the top of two statuettes handcrafted from ivory and from the horn of a rhinoceros of uncertain subspecies. Each specimen was sampled twice and the DNA from all samples was extracted using a phenol-based method. PCR was successfully performed followed by direct sequencing. To serve the needs of the typically degraded ancient DNA extracts, two sets of primers were designed to amplify short regions of the cytochrome-b gene. One primer set spans a 152 bp fragment which enables the discrimination between African and Indian elephants. The 192

bp amplicon generated by the second primer pair, allows the discrimination between the three subspecies of rhinoceroses. Species and subspecies identification was performed by analyzing the PCR-products through direct sequencing of the respective fragments. With respect to such subjects that have been handled extensively by humans during manufacturing processes and trading, both primer sets were designed to mismatch human DNA if the amplification is carried out under stringent conditions.

Results: All DNA extracts revealed amplifiable DNA. The newly designed primers generated highly specific products that enabled direct sequencing without problems. Control experiments showed that the primers did indeed not amplify human DNA as was expected from the experimental design. All sequence information was clearly readable from forward and reverse direct Taq cycle sequencing. From those sequences *Loxodonta africana* could be identified as the source of both statuettes. The investigation also revealed that the horn was derived from *Diceros bicornis*. By these species identifications it was shown, that the unambiguous analysis of strongly degraded DNA which is most likely contaminated though human DNA is possible.

Discussion: This PCR-based approach demonstrates the decisive role of DNA analysis in the future of conservation genetics and animal forensics. The method can be applied to a wide variety of questions exceeding mere species identification, targeting on establishing large databases of the genetic characteristics of animals of certain areas to enable observation and recognition of individual animals and their descendants. This study is also an example for the importance of an elaborate primer design, especially when investigating specimens that were extensively handled by humans, for it enables to exclude human DNA from being co-amplified.

Keywords: degraded DNA, species identification, conservation genetics

WIL-FP-03
Species Identification of the Brown Bear and the Asian Black Bear

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The brown bear (Ursus arctos) and the Asian black bear (Selenarctos thibetanus) are endangered species and are ranked among Appendix-1 species of the Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora. Products of both species, such as bear gall and paw, are often used illegally and involved in cases of poaching and illegal trade. In order to punish the criminals who destroyed wildlife resources, species identification of bear products is an important issue. Due to the loss of morphological characteristics organs, products or other remnants cannot be used for species identification by traditional methods.

Based on DNA differences, we designed two quick methods to deal with the above mentioned problems. The method is based on the comparison of the mitochondrial DNA (mtDNA) cytochrome b (Cyt b) sequences of the brown bear and the Asian black bear which are downloaded from GenBank. The following primers were selected by the software program DNASIS2.5: the common primer pair BF/BR

(BF: 5'–TCCTACACGAAACAGGATC–3', BR: 5'–GGATGTT-CTACTGGT TGTC–3') and two species-specific primer pairs, i.e. BrF/BrR (BrF: 5'–TTCTATTTGCCTACG CTATC–3',

BrR: 5'–CTCCAATTCATGTTAGTGTTAG–3') and BlF/BlR (BlF: 5'–CGAAACAGG ATCCAACAAC–3', BlR: 5'–TTATTCC-TCGTTTGGATG–3').

Subsequently PCR detecting systems for species identification of the brown bear and the Asian black bear were established. The PCR product obtained from the common primer pair BF/BR was 443 base pairs long and was used as a positive control, and as species discrimination marker after digestion with restriction enzyme. To discriminate between the brown and Asian black bear, the PCR products from BF/BR were digested by restriction enzymes Cfo I and separated by 5% polyacrylamide gel electrophoresis. The resulting brown bear DNA fragments consisted of a 97 base pair fragment and a 345 base pair fragment. The Asian black bear result, however, showed 3 fragments: a 97 base pair, a 160 base pair and a 185 base pair fragment. The results show that this PCR-RFLP method is very effective in discriminating between materials taken from the brown bear and the Asian black bear. The species-specific primer pair PCR can also identify the brown bear and the Asian black bear directly. The PCR products obtained from species-specific primer pairs BrF/BrR (for brown bear) and BlF/BlR (for Asian black bear), were respectively 198bp and 344bp long, and the black Asian bear fragment showed fragments 69 base pairs and 90 base pairs and 185 base pairs in length after digestion with Cfo I.

We compared various DNA amplification conditions and the robustness and specificity of the three primer pairs with 29 brown bear samples from 4 potentially different populations, and 43 Asian black bear samples from 5 potentially different populations, and with 67 samples of other 40 species. The usefulness of the identification techniques for the two bear species was tested with muscle, skin, gallbladder and hair, collected from different storage conditions. Our assays reach 100% efficiency and never produced false positives. The two methods were proven to be reliable and effective and are ready for application in case work. Furthermore, this approach might easily be extended to other species.

Keywords: Brown and Asian black bear, species identification, mtDNA

WIL-FP-04
The Tobacco Products Forensic Examination for Determination of Falsification Facts

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Recently the persecution of economic crimes related to internal market of Russia becomes actual. Tobacco products are often the objects of economic crimes. Detection of falsified tobacco products is an important task for police and for legal tobacco producers. Forensic examination is one of the means for the solution of this problem. Because of this the number of examinations is growls aimed both on comparison with original products and determination of common source of origin of the investigated products. This examination belongs to the "complex" type, which are made by different specialities experts which finally formulate the joint conclusion. The main task of the complex examination of tobacco products is to establish the set of properties, allowing to detect the sings of falsification. The sings could be: morphological and polygraphical characteristics, type and quality of packing and special marks, composition of raw material (its morphology, physical and chemical properties, elemental composition). The complex of these properties is evaluated the sample and the compared with original products to detect the sings of falsification and with other samples to find out the common source of origin of falsified products.

Keywords: Tobacco, Falsification, Examination