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WILDLIFE FORENSIC GENETICS – TOOL FOR CONTROL OF POACHING

Paule,L.[1], *Krajmerová, D.*[1] , *Romšáková, I.*[1], *Nedelová, L.*[1], *Slamečka, J.*[2]

Summary: Wildlife forensic genetics is an applied science that has emerged from conservation genetic research and forensic genetic practice to serve as an investigative tool in wildlife law enforcement. Presented paper deals with an outline of the the research project aimed at the elaboration of a set of mitochondrial DNA markers for identification of the ungulate game species – red deer, roe deer, fallow deer and muflon and two large carnivores – brown bear and lynx. The second aim of the research project is the design of microsatellite multiplex PCR reactions for the reliable identification of individuals. The increasing pressure of the enviromental crime endangers the sustainable use of game species as well as the survival of endangered species. With regard to the several international agreements concerning the species conservation and trade with endangered animal species, wildlife forensic genetics serve also as a tool for identification of endangered species and their origin and also the products originating from them.

Key words: forensic genetics, poaching, endagered species, CITES

Introduction

Development of genetic studies of wildlife species (game or protected ones) within the last two decades has only been possible by the support of molecular genetic analyses. The extent of conservation genetics applications was proportional with the development of individual biological disciplines as population genetics, phylogeography and phylogeny as well as molecular ecology and taxonomy. One of the conservation genetics applications is the development of molecular techniques suitable for identification of species and individuals for the purposes of wildlife forensic genetics. Wildlife forensic genetics deals with the proofs of identification of species, populations and relatives, and individuals. Its development was parallel, although much delayed, with human forensic genetics methods and applications. Within national and international legal frameworks for the conservation of biotopes and biodiversity (e.g. CITES), the forensic analysis of DNA became to be a key tool in the fight against environmental crime concerning wildlife (game and protected) species [1], [4], [7], [9].

Genetic identification of species is based on genetic DNA markers which are rather conservative within the species but discriminative between the species. In animals, the gene loci of mitochondrial DNA, *e.g.* cytochrome ***b*** and cytochromoxidase, subunit I (COI) are most frequently used. DNA sequencing was accepted technique used by the International Society of Forensic Genetics (ISFG) [2], [3], [8] and it was accepted as the procedure in forensic identification [11].

On the other hand, the genetic markers of nuclear DNA, e.g. microsatellites or fingerprinting (AFLP) due to their high polymorphism are frequently used for the identification of individuals within the species. From among the genetic markers of nuclear DNA, some of them are species-specific and can also serve for species identification.

In general, forensic geneticists work with various types of biological material, *e.g*. blood, soft tissues, bones, teeth, hair, saliva, urine or faeces. Some types of biological material (*e.g*. bones, antlers) contains low amount of DNAs, but is well preserved during many years and on the other hand soft tissues yield a lot DNA but due to presence of enzymes it degrade much faster if not stored properly.

Numerous international treaties on species protection or trade with protected species (e.g. CITES) bound signatory parties to active protection of species given in appendices, but also to the active legal control of trade with protected species. In illegal trade with protected species as well in poaching rather sophisticated methods have been used within the last decades, and accordingly similarly sophisticated methods should be used for detection of environmental crimes that can also be used in legal processes.

[1] Ladislav Paule, PhD, professor, Diana Krajmerová, PhD, post-doc, Ivana Romšáková, PhD., post-doc, Lucia Nedelová, PhD student, Faculty of Forestry, Technical University, Zvolen, Slovakia;
[2] Jaroslav Slamečka, PhD, associate professor, Animal Production Research Centre, Nitra, Slovakia;
Corresponding author: Ladislav Paule, Faculty of Forestry, Technical University, SK-96053 Zvolen, Slovakia; paule@tuzvo.sk; Phone: +421 45 5206221.

Material and Methods

Different types of samples: tissues, blood, bones, hair as well as traces that are frequently used in human forensic genetics are also used as experimental or proof material in forensic genetic analyses of wildlife. The forensic proofs aimed at the poaching cases are basically based on two steps – identification of the species and identification of individual identity. The forensic proofs aimed at the illegal trade with protected animals are usually based on the identification of the species or subspecies and/or the placement of the individual into the phylogeography pattern for identification the origin of the individual and its possible repatriation into the country (population) of its origin.

Methods of the DNA isolation should correspond to the well established isolation protocols for DNA isolation for samples with high or low DNA content and should also respect stages of DNA degradation. The most frequently used methods of the DNA isolation are: CTAB method for tissues, and different kits for specific types of biological material e.g. blood samples, bones, hair (e.g. Machery Nagel, Qiagen etc.)

Ideal procedure would be the use of numerous species-specific primers parallel identifying the species and individuals. Unfortunately, there is lack of such primers for wildlife species, since most of the primers for wildlife species are developed from the primers which have been used in the past for domestic animals (Bovinae, Suidae and Caprinae) and many of them work on two or more wildlife species (red and roe deer, domestic pig and wild boar, etc.)

The protocol for wildlife forensic applications should cover two steps – identification of species using mitochondrial markers and identification of individuals using microsatellites or other markers able to recognize individual variation. Some of the nuclear DNA markers could be species-specific and could also easily recognize the species, e.g. the primer G10P in brown bear, which could easily differentiate the samples originating from brown bear and from the other wildlife species.

The aim of our research is: (i) to elaborate a set of species-specific markers for species identification, and (ii) to develop a simple multiplex set of microsatellite loci with a high degree of polymorphism for identification of individuals. This research is aimed at development of multiplex-primer set for identification of red deer (*Cervus elaphus*), fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), mouflon (*Ovis musimon*), chamois (*Rupicapra rupicapra*), brown bear (*Ursus arctos*), lynx (*Lynx lynx*), hare (*Lepus europaeus*) and rabit (*Oryctolagus cunninculus*). The ungulates and two large carnivores are the most frequent subject of poaching. We have also added two other species which are common among poached species, but on the other hand their economical value is not so significant. They could, however, serve as the model species to investigate the presence of meet samples in processed meet and meet products.

The molecular markers used for species identification within our project are cytochrome ***b*** and cytochromoxidase, subunit I (COI) and D-loop. The number of tested microsatellites for individual ungulate or carnivore species depends on the species and varies at present from 12 at lynx, 17 at brown bear and red deer to 24 at chamois. Although, most of the primers amplify well, their suitability is based on the number of detectable alleles in general, and/or in specific population. In this respect, population-specific alleles could be important contribution when identifying the origin of unknown sample.

The development of simple multiplex set of microsatellite loci should therefore be aimed at the optimization of the number of loci (price) and the probability to detect an individual in a population. The primers with higher number of alleles are of advantage, while those with low number of alleles are of value only in case these alleles are species- or population- specific.

Results and Discussion

The most common cases in wildlife forensic genetics is poaching. From the methodological point of view, this is linked to the identification of the object, usually localized in the forest e.g. skin, intestines, blood traces, and the proofs at the subject e.g. meet, trophy, skin, etc. If both samples are available at the same time the molecular proof is rather simple and it is linked to matching the profiles or genotypes of both sets of samples. The number of markers used in the multiplex set increases the probability of correct identification.

More problematic are cases of poaching where the poacher or subject is not known. There are only identified traces and sampled biological proofs from the forest, but in the given moment the subject is unkown. As the ideal case would be the identification of biological proof (species, sex, genotype) and store the sample in the database for later comparison with other cases e.g. trophies, or meet products on the market etc. Development of the wildlife forensic database with „unsolved“ cases is considered as the good investment for the future. The development of

more precise protocols (more primers or different primers), the storage of DNA aliquots or biological samples would be of value. In this case the archived samples could be helpful to give more precise samples.

According to the valid legislation in Slovakia (Hunting Act), each legally hunted game individual (red deer, roe deer, fallow deer, mouflon, wild boar and brown bear) has to be equipped with a unique mark, the number of which has to be recorded in hunting licence of the hunter and the number of which has to accompany the animal to the end-user. Any hunted game individual without this unique mark is thus considered as a poached one, even if the hunter possesses the hunting licence.

Just for illustration several case studies we did in our lab:

- numerous proofs of identity between the biological traces of poached animals and the biological traces (meet, blood, trophies) at the subject in different species (red deer, roe deer, wild boar),
- proof of identity of brown bear hair remained on the killed person with the genotype of shot brown bear afterwards. This proof was necessary to validate the decision to remove the correct brown bear with untypical behaviour which killed a man,
- proof of identity of brown bear meat in the restaurant with legally culled brown bears.

The cases studies reported under the first two bullets used the comparison of two sets of samples using a higher number of markers. Thus the procedures were methodically rather simple, but due to the samples of the different quality and sometimes, due of unknown species, rather time consuming.

The third case study requires a database of all legally culled brown bears (or other species) meat of which could be put on the market (meat is property of the hunting unit, not of the hunter, and it could be marketize). According to the Slovak legislation, brown bear is considered as the protected species and the exceptions for annual quotas are issued by the Ministry of Environment. According to these exceptions the sample for genetic analysis and biometrical measurements of culled brown bear should be done by a person from the State Nature Conservancy. In this case, the complete set of samples of culled brown bears could be available for any further legal procedures.

Besides the species identification there is often the necessity of subspecies identification. Subspecies of chamois (*Rupicapra rupicapra tatrica*), Tatra marmot (*Marmota marmota latirostris*) are subject of nature conservation and strictly protected, while the nominate subspecies *Rupicapra rupicapra rupicapra* and *Marmota marmota marmota* are widely hunted as game species in the Alps. For both species, we have developed the methods for differentiating the subspecies which could easily serve as the proof on the subspecies level, whether the individual, chamois or marmot, has been poached in the High Tatras or it originates from the Alps. Similar case is also the capercaillie, which is freely hunted in Russia and Scandinavia and protected in most European countries. In these cases the forensic proofs are on subspecies level rather than on species level.

Another important subject of forensic applications is the control of the national and international trade with raptors used for falconry. All individuals kept in captivity and used for breeding purposes, should be genotyped and equipped with electronic chip which makes verification of the origin of their progenies (developing of pedigrees) possible. In other words, the trade with raptors is not forbidden, but the forensic applications should help to control the legal origin of individuals and prevent catching the animals from the wild populations.

Conclusion

Wildlife forensic genetics as a branch of conservation genetics has developed within the last 20 years. Its broader use is, however, limited for the countries with well-developed molecular science as well as environmental legislation paying significant attention to environmental crimes e.g. poaching, use of endangered and protected animal species and trade with endangered species and derived products from them.

According to our previous experience, the application of forensic techniques is possible also for the wildlife species for detecting the species and in many cases also subspecies from anonymous biological samples. Use of molecular markers based of nuclear DNA enables also to identification of individuals. It makes the proof of identity between biological traces in the forest and the biological material at the suspect (meat, trophy) possible.

Further development of wildlife forensic genetics requires the development of simple protocols identifying on one side species and multiplex set of primers for identifying individuals at considerably low price and high accuracy.

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