Theses of university doctoral (PhD) dissertation

Protecting wildlife and preserving the purity of hunting using forensic genetic methods in local ruminant game species

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1. Background and aims of the doctoral thesis

The protection of wildlife has been receiving increasing attention at both national and international levels, as various anthropogenic impacts – such as habitat reduction, the introduction and spread of non-native species, and illegal trade and poaching, which particularly affect endangered animals – pose significant threats to native fauna and ecosystems worldwide. The suppression, prevention, and mitigation of illegal activities involving wild animals, therefore, represent an ongoing global challenge.

In Hungary, hunting-related activities have developed into a major industry, generating substantial revenue for both local hunting associations and the national economy. Hungarian hunting grounds are frequented not only by local hunters but also by a considerable number of foreign guest hunters. This is evident by the fact that, in the previous year, approximately 30% of the four native trophy-bearing ruminant species were harvested by foreign hunters. Among Hungary's native wildlife, antlered species are particularly favoured hunting targets, and the trophies obtained from them are recognized internationally for their outstanding quality. Over the past decade, roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and fallow deer (*Dama dama*) antlers, together with mouflon (*Ovis aries musimon*) horns, have collectively constituted more than 90% of all trophies submitted for evaluation. Beyond their trophies, the hides and meat of these animals also represent significant value.

However, due to their considerable economic and symbolic value, these species are frequent targets of poaching and other forms of illegal exploitation. As Hungarian hunting enjoys a strong international reputation, maintaining the integrity and transparency of hunting practices is not solely

an ethical imperative but also a matter of legal importance. Deviations from statutory regulations typically entail legal consequences. In addition to considerations related to the preservation of natural habitats and biodiversity, economic factors – such as poaching, antler collection, and the illegal trade of animal products – may also give rise to criminal and civil proceedings. According to the Büntető Törvénykönyv, the Hungarian Criminal Code, poaching is defined as hunting without the appropriate permits (firearm or hunting licenses), outside of the hunting season, or through the use of prohibited tools or methods. Furthermore, hunting seasons vary among species and are often differentiated according to the age and sex of individuals. Numerous cases of unauthorized hunting activities are reported annually, which may result in severe penalties, including substantial fines, confiscation of equipment, or imprisonment of up to three years.

Within judicial proceedings, the role of genetic evidence has gained significant prominence due to its accessibility, reliability, and evidentiary value. Consequently, the development and implementation of appropriate forensic tools in this domain have become increasingly important. Forensic genetics, as an applied scientific discipline, employs molecular genetic techniques to analyse biological material. This approach provides an evidentiary framework for corroborating or refuting forensic hypotheses, thereby establishing or excluding a potential link to a criminal act. The application of advanced forensic genetic methodologies, coupled with the systematic organization of reliable genetic databases – analogous to those used in human DNA analysis – considerably broadens the range of cases that can be resolved, many of which were previously only partially or not at all solvable.

Although such genetic methodologies have been developed for several species, the existence of interspecific and population-level genetic differences often necessitates the testing of existing protocols or the development of new, species- or population-specific approaches. The objective of my research was to develop or locally validate genetic methods that could contribute to the resolution of forensic cases involving Hungary's four largest trophy-bearing species – the European mouflon, European roe deer, fallow deer, and red deer. To this end, my study sought to develop molecular approaches suitable for sex, species, and individual identification, and to evaluate the applicability of mitochondrial control region analyses for determining population origin, as well as for use in population genetic and exclusion analyses.

2. New scientific findings of the thesis

1. THESIS: The *DeerSex-plex* assay proved suitable for sex identification in local European roe deer, red deer, and fallow deer populations. Based on *in silico* analyses, it is also potentially applicable to other populations and closely related species.

DeerSex-plex was able to identify the correct sex in all tested samples. Primer binding regions were consistent with the SRY marker in red deer subspecies (*C. e. yarkandensis, C. e. maral*), wapiti (*C. canadensis*), white-lipped deer (*C. albirostris*), sambar deer (*C. unicolor*), sika deer (*C. nippon*), two subspecies of Eld's deer (*Rucervus eldii thamin, Rucervus eldii siamensis*), white-tailed deer (*Odocoileus virginianus*), reindeer (*Rangifer tarandus*), Chinese muntjac (*Muntiacus reevesi*), crested muntjac (*Muntiacus crinifrons*), Siberian roe deer (*Capreolus pygargus*), water deer (*Hydropotes inermis*), hog deer (*Axis porcinus*), and David's deer (*Elaphorus davidianus*). AmelX was conserved in wapiti, muntjac, moose (*Alces alces*), Japanese serow (*Capricornis crispus*), takin (*Budorcas taxicolor*), water buffalo (*Bubalis bubalis*), zebu (*Bos indicus*), and sheep (*Ovis aries*). For these species, the method may be applicable for sex identification following optimization of the annealing conditions.

2. THESIS: The *DeerSpec-plex* assay proved suitable for the species identification of domestic European roe deer, red deer, and fallow deer. Based on *in silico* analyses, it may also be applicable to other populations.

DeerSpec-plex correctly identified the species of all tested samples. Although one to four SNPs were detected within the binding sites of the cytB primers for European roe deer and red deer sequences in GenBank, no loss of specific

fragment amplification was observed during empirical testing.

3. THESIS: Analysis of mutation hotspots across the complete control region of fallow deer revealed that these regions vary among populations; consequently, the most informative section for analysis differs from country to country.

The number and distribution of variations within the control region depend largely on the geographical origin of the population. While mutational hotspots may occur within specific sequence segments in some regions, the same segments show little variation in others.

4. THESIS: A nationwide analysis of fallow deer haplotypes and their distribution identified a previously undetected sixth haplotype. Furthermore, the calculation of the random match probability (RMP) established a foundation for future exclusion-based analyses.

The RMP value calculated for domestic fallow deer (RMP = 0.547) indicates a relatively high probability of coincidental matches. This information is essential for the correct interpretation of genetic evidence in forensic investigations, as the strength of genetic evidence is directly influenced by the exclusion efficiency derived from local haplotype frequencies.

5. THESIS: In red deer and European roe deer, no significant differentiation among subpopulations was detected based on the mitochondrial control region, except for European roe deer carrying the Siberian roe deer haplotype, which occurred exclusively in the eastern part of the country.

For European roe deer, previous studies and the present findings indicate that analyzing only shorter segments of the control region results in considerable information loss. Therefore, it is advisable to amplify the entire control region – or at least both hypervariable regions I and II – in exclusion studies, particularly in forensic contexts.

6. THESIS: Of the 99 tetrameric microsatellite loci examined, 14 polymorphic markers were identified as suitable for the individual identification of fallow deer.

The combined probability of identity $P(_{ID})$ – the likelihood that two randomly selected individuals will exhibit identical genotypes based on the examined loci – was calculated as 5.2×10^{-5}. This indicates that approximately 5.2 out of 100,000 individuals would share the same genotype across the 14 selected tetrameric STRs. Considering that the Hungarian fallow deer population comprises approximately 40,000 individuals, this corresponds to roughly two individuals with identical genetic profiles across the entire population. This level of resolution meets international standards for individual identification in natural populations.

3. List of publications related to the PhD

1. Zenke P, Zorkóczy OK, Lehotzky P, Ózsvári L, Pádár Z (2022) Molecular sexing and species detection of antlered European hunting game for forensic purposes. Animals 12:246.

2. Turi O, Wagenhoffer Z, Battay M, Lehotzky P, Zorkóczy O (2023) Mikroszatellita-markerek tesztelése dámszarvasok egyedi azonosítása céljából. Magyar Állatorvosok Lapja 145:67–76.

3. Zorkóczy OK, Turi O, Wagenhoffer Z, Ózsvári L, Lehotzky P, Pádár Z, Zenke P (2023) A Selection of 14 Tetrameric Microsatellite Markers for Genetic Investigations in Fallow Deer (Dama dama). Animals 13:2083.

4. Zorkóczy OK, Wagenhoffer Z, Lehotzky P, Pádár Z, Zenke P (2024) Mitochondrial Control Region Database of Hungarian Fallow Deer (Dama dama) Populations for Forensic Use. Animals 14:1911.

5. Zorkóczy OK, Bujtor Z, Wagenhoffer Z, Lehotzky P, Zenke P (2024) Sejtmagi és mitokondriális genetikai markerek tesztelése hazai muflonokban (Ovis aries musimon). Magyar Állatorvosok Lapja 146:615–624.