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# Applicability of degraded biological remains in conservation genetic studies of birds

Case study of the Eastern Imperial Eagle (*Aquila heliaca*) population of the Carpathian Basin

**Brief Summary of Doctoral Thesis**

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## Introduction

The number of conservation studies in which molecular methods are used is increasing. The aim of these researches is mostly to compare two populations of the same species, to investigate their individual or population sized relationships and to estimate their genetic stability. However genetic sampling of some species can be difficult, but as the molecular methods got more sensitive it is now possible to use non-invasively collected samples like hair or feathers for these studies. Such samples are usually of poor quality and the extracted DNA is very fragmented. At the same time this type of sampling for conservation biology studies is very favourable, especially when studying endangered or stress sensitive species. In bird studies feather colleting is feasible if the nesting sites are known and can be carried out simultaneously with the monitoring of the species without additional field work.

DNA extracted from non-invasively collected feathers is usually fragmented and of low quality. As there is a known uncertainty in the data that can be obtained from such samples it was important to determine how much difference is between the quality of DNA extracted from feathers and from the traditional or invasively collected samples. Usually the feather tip is used as DNA source, and it was confirmed that in this case the physical state of the feathers is the most important factor, but the feather type has no effect.

In this research the blood clot remaining at superior umbilicus part of the feather shaft was used as DNA source. This method is

highly cited but rarely used because it usually requires relatively big feathers. Thus the methodological aims of this research were to:

1. compare the quality of DNA extracted from non-invasively collected samples (further on referred to as “feathers” or “feather samples”) with those extracted from minimal invasively collected blood samples (further on referred to as “blood” or “blood samples”);
2. determine to what extent the physical state (quality, type, size and age) of the feather influences the quality of the extracted DNA;
3. determine whether some parameters can refer to the degree of DNA fragmentation.

For this feather samples of Eastern Imperial Eagles (*Aquila heliaca*) were used. Feathers were collected in the Carpathian Basin by the staff of MME BirdLife Hungary and the Raptor Protection of Slovakia. Blood samples were taken on special filter paper by the staff of RPS.

Until the end of the 1980's this Eastern Imperial Eagle population resided in small number in the North Hungarian Mountains. As at the start of the 1990's hunting them became illegal and simultaneously the species conservation activity begun the population started to expand. This provides a unique opportunity to study the expansion of an endangered species, and also the population genetic and population dynamical effects of this expansion. Thus with the use of the eagles’ DNA samples we approached the following questions:

1. Are the sampling method and the available marker set appropriate for population genetic and population dynamic studies of the imperial eagles in the Carpathian Basin?
2. Can the imperial eagle population in Slovakia be regarded as continuous? Is there any evidence of gene flow between the eagles residing in the eastern and western parts of the country?
3. What is the annual mortality rate of the eagles or if this cannot be studied, the annual turnover rate of the population in the eastern part of Hungary?
4. Can the methods used for territory mapping by the staff of the MME follow the changes in the territory system despite of the expansion of the population?
5. What patterns can be observed in the territory choice of the imperial eagles?

## Methods

### Sample collection and DNA extraction

Shed feathers of eagles were collected by the staff of MME BirdLife Hungary and the staff of Raptor Protection of Slovakia (RPS) in June and August from 1997 to 2006 under the nests during the ringing of the chicks. Apart from this more shed feathers from adults and blood samples from chicks were collected in Slovakia by the RPS on special filter paper.

Feathers were categorized according to their physical state. Those with no damage to the superior umbilicus or the feather shaft were classified as good, others with slightly damaged superior umbilicus and shaft were categorized as abraded and feathers with pronounced damage were ranked as poor.

All feather shafts were cleaned with ethanol before cutting out the superior umbilicus with a sterilised scalpel. To facilitate the digestion DTT (1,4-dithio-D-threitol) was given to each feather sample. All blood samples were processed according to the manufacturer's protocol.

### Molecular sexing

Eagles are sexually monomorph with a small difference in size thus the sex-chromosome linked CHD1W and CHD1Z genes were used for molecular sexing. The used primers (2550F/2718R) amplify parts of the CHD1 gene that contain introns that that differ in size in the W and Z chromosomes. The exact length of the products differs in each species (W: 400 – 450 bp, Z: 600-700 bp), but the

difference is big enough to separate them by electrophoresis on 2 – 2.5% agarose gels.

**Microsatellite based individual identification**

Individual genotypes were determined by two dinucleotide (Aa02 and Aa39) and six tetranucleotide (IEAAAG04, IEAAAG12, IEAAAG11, IEAAAG15, IEAAAG09 és IEAAAG14) microsatellite loci. The exact fragment lengths were determined by capillary electrophoresis on an ABI PRISM® 310 and an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems) with the softwares GeneScan 3.7 and PeakScanner 1.0 (Applied Biosystems). All genotypes were assigned blind as each sample got a tag independent from its origin. Consensus genotypes were assigned after three independent readings (Szilvia Kovács, Krisztián Szabó, Nóra Vili).

**Calculations and programs**

Data entry errors were checked with the "MS Microsatellite Toolkit". The occurrence of null alleles and possible genotyping errors were estimated with the "MICRO-CHECKER 2.2.3". Probability of Identity and Mantel tests were calculated with "GenAlEx 6.41" and "zt 1.1". Deviation from the Hardy-Weinberg Equilibrium and the probability of Linkage Disequilibrium between the loci was calculated with the online version of "Genepop 4.0.10". Statistical analyses were carried out with the "R v. 2.12.1".

**Calculation of the turnover rate**

Due to incomplete data sets caused by failed sampling or genetic tagging, the factual events of bird changes could be stated

only with uncertainty. Therefore minimum and maximum rates of yearly bird turnover were calculated. According to complete sequences of genetically tagged feather samples, it is highly unlikely that a previously identified bird disappears and is later found in the same territory as no such events were recorded. Based on this, no change in territory was assumed when the same bird was identified before and after the missing year or years. When another individual was found after the hiatus, minimum and maximum turnover rates were computed according to the number of missing years. Turnovers were counted in each territory then averaged to obtain the minimum and maximum average turnover rates.

## Results

### DNA Quality

Altogether 717 feathers and 126 blood samples were analysed and 87.8% of all samples yielded DNA suitable for molecular sexing. There was no significant difference between the two types of samples (feather samples 85.5% and blood samples 98.4%). We found significant difference between the amount of feather samples derived from females (90.6%) and from males (9.4%). In the case of the chicks' samples this ratio did not differ between females and males (48.8% and 51.2% respectively).

To test the effects of feather quality, type and age feathers with enough background data could only be used, this means 484 good quality feathers, 81 abraded feathers and six poor quality feathers were examined. 86.8% of good quality feathers, 81.4% of abraded feathers and 33.3% of poor quality feathers yielded evaluable PCR-product after the amplification of the CHD1 locus.

The effects of physical state were analyzed with Conditional Inference Tree method and logistic regression. According to our results the efficiency of the amplification of the CHD1W/Z gene was influenced significantly by feather quality and we found significant interaction between feather quality and feather type. However according to our results neither this nor the time passed between feather collecting and DNA extraction had effect on DNA quality.

### Data quality and sensitivity of the marker set

There was no indication of stuttering or allelic dropout on any of the loci. However locus IEAAAG09 and locus Aa39 was suspected

to contain null-alleles. In order to avoid bias caused by this only the heterozygous genotypes on these loci were taken into the analyses. There was no deviation from either the Hardy-Weinberg Equilibrium or from the Linkage Equilibrium on the other six loci separately and examining them all together. Probability of Identity was $3.7 \times 10^{-6}$, small enough to use the marker set for individual identification.

**Genetic structure of the Imperial Eagle population in Slovakia**

The studied Imperial Eagle population is divided into two by geographic barriers (approximately 150 km). We found marginally significant difference between the Eastern and Western subpopulations based on the DNA-profiles determined by microsatellite genotypes and haplotype determined by mtDNA sequence analyses (study of Szilvia Kovács).

**Validation of the territory mapping method**

The DNA profiles of individuals from altogether 46 territories were determined. This means that approximately 60% of the active nest sites were sampled. During the study females (pairs) used the same nests 20 times, and nesting sites were changed in 42 cases. All genotyped individuals stayed in the territories defined by field methods or disappeared, meaning there was no case where the genetic identification contradicted the results of the territory mapping.

**Yearly average turnover of the females**

The minimum average annual turnover rate of females was 27.2% based on 153 genotyped individuals, while the maximum rate was 35.5%. The turnover rate in the mountain territories seems to be

smaller than in the lowlands (21.2-28.3% vs. 29.3-38.0% respectively) although the difference was not statistically significant.

**Pattern of the expansion**

In this study 108 nesting events were studied. Most of the new territories were established between the older ones, but several nests were found in considerable distance (more than 90 km) from other nests. The distribution of the nest distances was bimodal, not normal and continuous, so we assume there is no uniform strategy for choosing the nesting sites.

In order to determine whether there is a correlation between the location of the nests and relatedness of the individual (i.e. closer related individuals tend to move less far) Mantel tests were run in the case of nests where both genetic and geographic data was available. According to the results there was no correlation between the geographic distance and the degree of kinship. Meaning the observed pattern of the territories could not be explained by the relatedness of the individuals.

## Discussion

### Effects of sample type and physical state of the feathers

We found no difference in the amplificability of the DNA extracted from the superior umbilicus of the feathers and from minimal invasively collected blood samples. Furthermore, similarly to other previous studies investigating the relationship between feather quality and DNA quality if DNA was extracted from the feather tip, we found that only the physical state of the feathers had significant effect on DNA quality if the superior umbilicus was used as DNA source. However if abraded feathers were used greater coverts, secondaries and tertials yielded typically worse quality DNA than other feather types, although the difference was not significant.

According to our results good quality and abraded shed feathers, if stored in dark and dry conditions yield DNA that can be used for individual based population genetic studies of Imperial Eagles. Feathers collected in the field can be sent to the laboratories reducing the costs like travelling, sample storing or adjusting the methods.

### Genetic structure of the Imperial Eagle population in Slovakia

The Imperial Eagle population in Slovakia is divided by geographic barriers. The distribution of microsatellite allele frequencies and mtDNA control region haplotypes suggest a small genetic difference between Imperial Eagle subpopulations in East and West Slovakia. However if examining the whole population of the Carpathian Basin the results suggest that the Carpathian Basin's breeding birds should be handled as one uniform population. The

western and eastern breeding nuclei in Slovakia can be handled as subpopulations, with slightly limited gene flow among them.

**Validation of the territory mapping method**

In a previous study of a stable imperial eagle population in Kazakhstan high territory and mate fidelity was found. That is they found no evidence of territory exchange between residents and no extra-pair offspring were found. The population in the Carpathian Basin is expanding therefore we checked whether the used territory mapping method can follow the changes in the territory system of this population.

There was no contradiction between the result of genetic tagging and the territory marked as the origin of the feather. That is feathers of re-identified individuals consistently belonged to nests which were assigned to the territories in which the individuals were tagged for the first time. Altogether two cases of territory swapping occurred, but in these cases the two original territories were left empty.

Based on our study, the territories are existing units whose basic position does not change even if the resident pair disappears. According to the results the used territory mapping method is appropriate for identifying and assigning newly built nests into new or already existing territories.

**Yearly average turnover of the female breeding birds**

We determined the minimum and maximum yearly average turnover rate because not all samples from all years yielded evaluable PCR products. The minimum rate was 27.2% and the

maximum rate was 35.5%. These rates are much higher than it could be expected in an expanding population and what, according to several publications is not typical for long lived raptors (6-12%). In a recent study of a stable Imperial Eagle population in Kazakhstan the average yearly loss was 16%.

In the last few years besides the mortality caused by electrocution, the intentional or accidentally caused poisoning is a serious threatening factor. However carcasses are rarely found and so only the absence of genotyped individuals can be surely detected.

The fact that despite this high turnover rates the studied population is still expanding, means that there is still space available for new pairs in the Carpathian Basin. Besides this we can assume the existence of a large number of floater individuals that can fill up the vacant territories.

**Pattern of the expansion**

At the start of the 1990's the human disturbance decreased and the Imperial Eagle population in the Carpathian Basin started to expand towards the lowlands. The expansion started slowly but later continued exponentially. After the study of the nests' position throughout the years, we found that the majority of the new breeding pairs tend to establish new territories between the already existing ones. However some (10 cases in 27 years) of the new pairs start to breed far (even 90 km) from the inhabited areas. The empty spaces are later filled up by new pairs.

Neither the nearest nest distances between new and old territories nor the logarithm of the distances was continuous, so we assume there are two strategies present in the population. One of

them is that new pairs establish their nests in an area which is already used by eagles. This probably means smaller risk for the pair but also smaller gain. The other strategy is that pairs choose a territory far from the other territories, which means they take a bigger risk, because it is not sure that the location will be suitable for nesting. However their gain can be bigger since they can defend the territory with less effort and, if enough food is available chicks can grow up with greater probability.

The expansion started when only a few breeding pairs existed in Hungary, so it was possible that the pattern of expansion reflects the eagles' relationships. This was however disproved by the result of the Mantel-tests of genetic and geographic distances, as no correlation was found.

It is possible that immature individuals can prove the suitability of the areas in which they spend time before they settle down. This assumption was previously proven for the Spanish Imperial Eagle. This and the natal philopatry of the species are currently being investigated. According to preliminary results only very small portion of the chicks comes back and breed in the Carpathian Basin. More than 200 chicks, which fledged between 2004 and 2007, were genotyped so far, but only 12 of them were found in the 2011 breeding population. Thus for the present we could not perform statistical analyses of the distances between fledging and breeding.

## New scientific results

### Methodological results

1. The comparison of the quality of DNA extracted from shed feathers and from samples taken with minimal invasive methods showed no difference in applicability.

2. The comparison of the feathers' physical state (quality, type, age and size) and the quality of extracted DNA showed that the quality of the feathers is the primary indicator of the fragmentation of DNA if its source is the superior umbilicus.

3. We could prove that the methods and markers are appropriate and sensitive enough to use for population genetic studies.

### Population genetic and population dynamic results

1. Based on nuclear and mitochondrial markers there is limited gene flow between the eastern and western Imperial Eagle subpopulations in the Slovakian population.

2. We determined the minimum (27.7%) and the maximum (35.5%) yearly average turnover of female Imperial Eagles in the Carpathian Basin.

3. Based on our results of genetic tagging the traditionally used territory mapping method is suitable to follow the changes in an expanding population.

4. According to the analysis of the expansion pattern of the population there are two different strategies for establishing territories present. The expansion is not constant as new pairs usually nest in between or near to existing ones, but some are established in a greater distance and are later surrounded by newer territories.

5. Based on the correlation analysis of genetic and geographic distances the observed pattern of expansion is not corresponding to the kinship relations of the birds.

6. The established a database and gene bank is available for future studies on population genetics and natal philopatry of the population.

## Publications related to the dissertation

Vili N., Kalmár L., Kovács Sz., Horváth M.: **Parlagi sasok (*Aquila heliaca*, Savigny, 1809) populációgenetikai vizsgálata a Kárpát-medencében**, In: Forró L. (ed.): A Kárpát-medence állatvilágának kialakulása. [The genesis of the fauna of the Carpathian Basin] Magyar Természettudományi Múzeum, Budapest pp. 303-310. ISBN: 963-7093-99-9, 2007

Vili N., Horváth M., Kovács Sz., Chavko, J., Hornung E., Kalmár L.: **Alternatív mintavételi módszer gyakorlati alkalmazhatósága madárgenetikai vizsgálatokban: parlagi sasok ivarmeghatározása, mikroszatellitákon alapuló egyedi azonosítása és mtDNS-ének vizsgálata.** Magyar Állatorvosok Lapja 131: 426-435., 2009
$IF_{2009}$: 0,200

Vili N., Horváth M., Szabó K., Kovács S., Chavko J., Hornung E. , Kalmár L.: **Genetic structure of the Imperial Eagle (*Aquila heliaca*) population in Slovakia.** Slovak Raptor Journal 3: 21–28, 2009

Vili N., Nemesházi E., Kovács Sz., Horváth M., Kalmár L., Szabó K.: **Factors affecting DNA quality in feathers used for non-invasive sampling.** Journal of Ornithology, 2013, doi: 10.1007/s10336-013-0932-9
$IF_{2011}$: 1.636

Vili N., Szabó K., Kovács Sz., Kabai P., Kalmár L., Horváth M.: **High turnover rate revealed by non-invasive genetic analyses in an expanding Eastern Imperial Eagle population.** Acta zoologica Academiae Scientiarum Hungaricae, in press

$IF_{2011}$: 0,564

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