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Genetic structure of the Imperial Eagle (*Aquila heliaca*) population in Slovakia

Genetická štruktúra populácie orla kráľovského (*Aquila heliaca*) na Slovensku

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Abstract: The distribution of the Imperial Eagle (*Aquila heliaca*) in the Carpathian Basin is not continuous, since western and eastern breeding pairs are separated by 150 km from each other in Slovakia, and 70 km in Hungary. In the present study our aim was to examine whether this geographical distance has resulted in any genetic separation between the Western and Eastern Slovak breeding groups. We have used 132 shed feathers and 128 blood samples collected in the fields geographically representing the whole of the Slovak breeding population, and included all juveniles ringed between 2004 and 2006. After successful DNA extractions we have determined the sex, microsatellite DNA-profiles and mtDNA control region haplotypes of the specimens. Data were integrated in a common Hungarian-Slovak "DNA-fingerprint" database, making identification of the same specimen possible when recaptured. Based on a subsample of the collected individuals, the genetic structure of the Slovak population was tested using ten microsatellite loci and mtDNA control region haplotypes, and marginally significant genetic differentiation was found between western and eastern subpopulations. These results suggest that, in spite of the large dispersal capacity of the species, a relatively small geographic distance can also decrease the exchange rate of individuals between subpopulations. As this result involves only samples from the northern part of the breeding area, major conclusions concerning genetic structure and gene flow of Imperial Eagles in the entire Carpathian Basin population cannot be drawn without sampling and analysing the southern subpopulations in Hungary.

Abstrakt: Rozšírenie orla kráľovského v Karpatskej kotline nie je súvislé. Páry hniezdiace na západnom a východnom Slovensku sú od seba vzdialené 150 km a v Maďarsku 70 km. Cieľom predkladanej práce je zistiť či táto geografická vzdialenosť spôsobuje genetické odlišnosti medzi západoslovenskými a východoslovenskými pármi. Použitých bolo 132 vzoriek z pier a 128 krvných vzoriek orlov získaných z územia pokrývajúceho celú hniezdnu populáciu na Slovensku, zahrňujúc krvné vzorky od všetkých juvenilných orlov krúžkovaných v rokoch 2004–2006. Po úspešnej extrakcii DNA bolo zistené pohlavie jedinca, profil mikrosatelitovej DNA a haplotyp kontrolného regiónu mitochondriálnej DNA. Údaje boli zjednotené do spoločnej maďarsko-slovenskej "DNA-fingerprint" databázy, ktorá je použiteľná v prípade identifikácie znovukontrolovaného jedinca. Genetická štruktúra slovenskej populácie bola testovaná za pomoci 10 mikrosatelitových lokusov a haplotypov kontrolného regiónu mtDNA. Medzi západoslovenskou a východoslovenskou subpopuláciou bola zistená marginálne signifikantná genetická odlišnosť. Tieto výsleky poukazujú na to, že i napriek veľkej rozptylovej kapacite druhu, aj relatívne malá izolovanosť môže zapríčiniť spomalenie výmeny jedincov medzi subpopuláciami. Predkladané výsledky zahŕňajú iba vzorky zo severnej časti hniezdneho areálu druhu, preto závery týkajúce sa genetickej štruktúry a toku génov v rámci celej populácie orla kráľovského v Karpatskej kotline nemôžu byť opísané bez analýzy vzoriek južnej subpopulácie v Maďarsku.

Key words: feather, non-invasive sampling, non-destructive sampling, sexing, microsatellites, mtDNA

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Introduction

The Imperial Eagle (*Aquila heliaca*) is a globally threatened raptor species; it is listed as "vulnerable" by the IUCN. Its distribution area covers Eastern-Eurasia from the Carpathian Basin to the Baikal Lake. The world breeding population of the species is less than 10.000 pairs, out of which 140 pairs can be found in the Carpathian Basin (Horváth, Chavko & Wichmann, unpubl.). Its distribution in the Carpathian Basin is not continuous, since eastern and western breeding pairs are separated by 150 km from each other in Slovakia, and 70 km in Hungary. There are several records of immature birds, which have moved between the two breeding areas during dispersal (Danko 1996, Horváth & Kovács 2009), although according to field observations there is no confirmed data on any breeding bird, which fledged in one subpopulation and started to breed in the other.

The rapidly developing genetic methods, like analysis of microsatellite loci in nuclear DNA or analysis of mitochondrial DNA, have the possibility to answer the questions concerning genetic structure, population subdivision, or gene flow via migration.

Microsatellites are polymorphic loci in nuclear DNA, consisting of 2–6 base pairs long repeating units. They are selectively neutral, co-dominant and are used as molecular markers for wide-ranging applications in the field of genetics, including kinship and population studies (Chambers & MacAvoy 2000). Microsatellites owe their variability to an increased rate of mutation compared to other neutral regions of DNA. These high rates of mutation can be explained most frequently by slipped strand mispairing (slippage) during DNA replication on a single DNA double helix (Levinson & Gutman 1987). Owing to the high variability of these regions, we can obtain individual profiles of the observed birds by the simultaneous use of several loci.

Mitochondrial (mt) DNA has special characteristics that make it a useful marker for phylogenetic and population biology studies. These are maternal inheritance, lack of recombination, homoplasmy and a relatively high mutation rate. MtDNA control region is a useful and common marker for assessing genetic relationships of populations within a species despite the relatively low genetic variability (haplotype diversity).

Sample collection for genetic studies, can be a serious problem in the case of most wild, endangered or stress sensitive species. According to Taberlet et al. (1999) there are three groups of sampling methods: (i) invasive sampling (mostly used for invertebrate species, since animals need to be killed to get samples from them), (ii) non-destructive sampling (biopsy or blood sample, for which animals have to be caught), (iii) non-invasive sampling (using remains left after animals, e.g. shed feathers, hair, scat). Non-invasive sampling, when no disturbance is caused for the animals, is ideal for protected vertebrate species, although in some cases invasive and non-destructive sampling can also be used.

In the present study we attempted to reveal the genetic structure of the Imperial Eagle population in Slovakia, using non-invasively collected feathers of breeding adults and non-destructively collected blood samples of ringed chicks.

Material and methods

Sample collection and DNA extraction

Shed feathers of adult breeding birds (n=132) were collected under nest sites by members of the Raptor Protection of Slovakia (RPS), representing almost the whole breeding population in Slovakia. From 2004 to 2006, blood samples were taken from chicks (n=128) during the ringing procedure.

To retrieve DNA from the feathers, we used the method described by Horváth et al. (2005). The narrow channel found at the superior umbilicus part of the feather shaft contains a blood clot which is a remnant of a nutritive artery during the feather development and it proved to be a suitable source of DNA for genetic analyses. We cut out these clots with sterile blades to avoid cross-contamination of the samples.

All samples were stored in a freezer until DNA extraction, which was carried out according to the stan-

dard chloroform-isoamylalcohol method based on the description of Gemmel & Akiyama (1996) with minor alterations.

Blood samples were collected from the chicks' brachial vein using IsoCode STIX™ paper, which is a special impregnated paper that can be easily used in the field. The stress caused by the collection of blood samples can be reduced significantly with this method, since it was designed for properly storing a very small amount of blood (1–2 drops, equal to 10–12µl) especially for genetic examinations. DNA extraction from the paper was then performed following the manufacturer's protocol (CosmoBio, Schleicher & Schuell Biosciences). DNA extraction efficiency and DNA sample quality was checked by agarose gel electrophoresis (3µl DNA solution, 0.8% agarose gel).

Molecular sexing

As there is only a small sexual dimorphism in size we applied molecular sexing to identify whether the feathers derived from the male or female bird of the given territory. Molecular sexing was also applied for blood samples of the chicks, since there were no available data on the accuracy of the morphological sexing in the field. We used the PCR conditions (with minor alterations) and the primers (2550F and 2718R) developed by Fridolfsson and Ellegren (1999). These primers are specific for the CHD1 gene, amplifying a larger intron sequence (CHD1Z, 700bp) in both sexes and a smaller one (CHD1W, 450bp) in females only (Horváth et al., 2005). The two variants can be separated by agarose gel electrophoresis (5µl of the PCR product was visualized by ethidium-bromide on 2.5% agarose gel).

DNA-fingerprinting

There are 17 polymorphic microsatellite loci published for the Spanish Imperial Eagle (*Aquila adalberti*) and the Eastern Imperial Eagle. Nine of them contain dinucleotide repeats (Martínez-Cruz et al. 2002), while eight have tetranucleotide repeats (Busch et al. 2005). Out of these 17 loci, we have selected the most variable three dinucleotide (Aa35, Aa39 and Aa02) and seven tetranucleotide loci (IEAAAG04, IEAAAG12, IEAAAG11, IEAAAG13, IEAAAG1, IEAAAG09, and IEAAAG14). All loci were amplified with fluorescently labelled primers, using HEX (yellow), TET (green) and 6-FAM (blue) dyes.

PCR reactions were conducted as described by Martinez-Cruz et al. (2002) with some modifications. Touchdown PCR was performed in all loci, according to the TM values (melting temperature) of the primers: in five loci (Aa35, Aa39, Aa02, IEAAAG09 and IEAAAG14) annealing temperature was decreased from 66 to 50 °C, and in the other five ones (IEAAAG04, IEAAAG12, IEAAAG11, IEAAAG13 and IEAAAG15) it was decreased from 60 to 50 °C.

The exact fragment lengths were determined by capillary electrophoresis (ABI 310 automated sequencer) with the GeneScan 3.7 and the PeakScanner 1.0 softwares (Applied Biosystems), using the ILS 600 (Promega) internal lane standard.

Deviations from the Hardy-Weinberg equilibrium, allele frequencies and probability of identity (PI, the probability that two individuals drawn at random from a population will have the same genotype at multiple loci, Waits et al. 2001) were calculated with GenAlEx (Peakall & Smouse 2006).

mtDNA haplotype analysis

A region of 345 bp of the hypervariable Domain I of the mitochondrial control region (CR) was amplified with the primers AID1 (Martínez-Cruz et al. 2004) and Fbox (Godoy et al. 2004) using the authors' protocols.

CR products were purified with the Ultrafree-DA centrifugal filter (Millipore), and sequenced in both directions using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI 310 capillary sequencer (Applied Biosystems). DNA sequences were edited using the Staden software (Staden 1996) and aligned by hand.

Standard diversity indices, such as haplotype diversity (h) and nucleotide diversity (π), were determined using the DnaSP 4.10 software (Rozas et al. 2003). To reveal the relationships among the newly found and published Imperial Eagles' haplotypes, median-joining network (Bandelt et al. 1999) was constructed using the software NETWORK v.4.5.1. (Fluxus Technology Ltd. at www.fluxus-engineering.com).

Genetic differentiation between the two Slovak subpopulations was analysed using AMOVA (in both microsatellite and mtDNA CR sequences), performed with GenAlEx6 and Arlequin 3.11 (Excoffier et al. 2005). In mtDNA CR data, the non-parametric method of Raymound & Rousset (1995) was also analysed with Arlequin 3.11. Standard statistical tests were performed by the R 2.8 statistical program package (R Development Core Team 2007).

Results

DNA-extraction

Among the 260 Slovak samples examined (132 shed feathers and 128 blood samples), 85% of the feathers

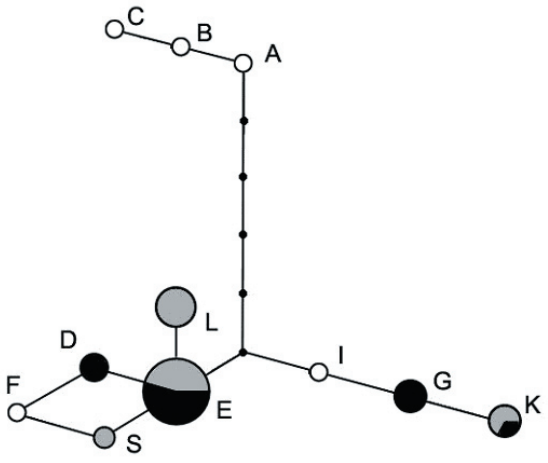

Fig. 1. Median-joining haplotype network of haplotypes found in Slovak Imperial Eagles, along with the published haplotypes of eastern (D, E, F, G, I) and Spanish (A, B, C) Imperial Eeagles (Martínez-Cruz et al. 2004). The size of the circles indicates the relative frequency of the corresponding haplotype in the Slovak population. Grey and black colours represents the frequency of alleles found in the eastern and western subpopulations respectively. Haplotypes with empty circle was not found in this study. Missing haplotypes are marked with a dot.
Obr. 1. Haplotypová sieť (median-joining) zistená u slovenských orlov kráľovských spoločne s publikovanými haplotypmi západných (D, E, F, G, I) a španielskych (A, B, C) orlov kráľovských (Martínez-Cruz et al. 2004). Veľkosť kruhu označuje relatívnu frekvenciu príslušného haplotypu v slovenskej populácii. Sivá a čierna farba reprezentuje frekvenciu alel vo východnej, resp. západnej subpopulácii. Haplotypy s prázdnymi krúžkami neboli zistené. Chýbajúce haplotypy sú označené bodkou.

and 98% of the chicks' blood samples yielded DNA of usable quality and quantity to perform the molecular sexing. There was no significant difference between the efficiency of the DNA-extraction from the feathers and the chicks' blood samples (Chi-square test, $\chi^2 = 0.894$, $P = 0.3443$).

Molecular sexing

In adult feather samples, 92% of the sexing procedures yielded a rateable result with 8 (7%, males) to 105 (93%, females) ratio. The sex ratio among the chicks was 64 (males) to 61 (females), which did not differ significantly from the 1:1 ratio neither considering all years nor year by year (Table 1). We also compared the results of sexing procedures carried out by morphological features of the hatchlings in the field to the outcome of the molecular method. We found that the sex of 21 chicks (68%) out of the 31 was correctly identified.

Tab. 1. Sex ratio of nestlings according to the molecular sexing procedure (M – male, F – female)
Tab. 1. Pomer pohlaví mláďat na základe molekulárnej analýzy (M – samec, F – samica)

year / rok	M	F	sex ratio / pomer pohlaví (M:F)	P*
2004	15	24	1:1.6	0.199
2005	23	12	1.91:1	0.089
2006	26	25	1.04:1	1
total / spolu	64	61	1.04:1	0.858

DNA fingerprinting

The DNA profile of 43 eagles was defined by the analysis of seven tetranucleotide and three dinucleotide microsatellite loci. Only a small proportion (17%) of the Slovak feather samples provided sufficient DNA-profiles. On the other hand, blood samples of the chicks yielded much better results, and the ratio of successful amplifications was 71%, therefore we gained enough data to analyse the fragment lengths of microsatellites of the eastern and western populations (E: 21 birds, W: 22 birds). The PI value of our microsatellite marker set was 6.8×10–8.

The average allele number per locus was 3.28 (tetranucleotide loci) and 7.66 (dinucleotide loci). Significant difference from the Hardy-Weinberg equilibrium was found at two loci (G09 and Aa39) in the eastern population and at two different loci (G04 and G15) in the western population.(Table 2 and 3).

MtDNA control region haplotypes

Owing to the low efficiency found at DNA-fingerprinting of feather samples, we selected the samples for mtDNA analyses mostly from blood samples. Altogether 11 non-sib nestlings (i.e. belonging to different broods) from East and 12 from West Slovakia were analysed, and a single feather sample from an East Slovak nest site was also involved in mtDNA analyses. We successfully determined the mtDNA control region sequences of all 24 specimens and identified four variable sites, defining six different haplotypes (D, E, G, K, L and S, Table 4.). The newly found three haplotypes (K, L and S) are closely related to those that were described from Kazakhstan (D, E and G, Martínez-Cruz et al. 2004), each differing in only one sequence position from them.

Tab. 2. Observed allele lengths and frequencies of the microsatellite loci involved in the study. Allele lengths (bp) are marked with bold; their frequencies in the eastern (E) and western (W) population are separated with a slash

Tab. 2. Pozorovaná dĺžka a frekvencia alel mikrosatelitových lokusov zahrnutých do štúdie. Dĺžka alel (pb) je vyznačená tučne; frekvencie vo východnej (E) a západnej (W) opulácii sú oddelené lomítkom.

locus / lokus	G04	G09	G11	G12	G13	G14	G15	Aa02	Aa35	Aa39
	218	**476**	**106**	**124**	**236**	**192**	**306**	**137**	**249**	**180**
	0/0.023	0.062/0.036	0.405/0.364	0.286/0.216	0.042/0.062	0.214/0.205	0/0.025	0.048/0	0.117/0.125	0.063/0
	228	**484**	**114**	**128**	**240**	**196**	**324**	**139**	**251**	**182**
	0.036/0.023	0.125/0.095	0.095/0.136	0.214/0.284	0.208/0.188	0.286/0.295	0.071/0.175	0.048/0.095	0.183/0.100	0/0.033
	232	**488**			**244**		**328**	**141**	**253**	**184**
	0.024/0.091	0.313/0.369			0.25/0.25		0.155/0.088	0/0.012	0.067/0.125	0.021/0.117
	236						**332**	**143**	**255**	**188**
	0.416/0.352						0.071/0.025	0.095/0.178	0.017/0	0.021/0.050
	240						**336**	**145**	**257**	**190**
	0.024/0.011						0.202/0.175	0.154/0.036	0.033/0.075	0.083/0.017
							344	**150**	**259**	**192**
							0/0.013	0.143/0.143	0.083/0.075	0.125/0.100
								152		**193**
								0.012/0.036		0/0.050
										195
										0.083/0.133
										197
										0.041/0
										199
										0.063/0
∑ (E / W)	**21/22**	**20/21**	**21/22**	**21/22**	**6/4**	**21/22**	**21/22**	**21/21**	**15/10**	**12/15**

Tab. 3. Expected and observed heterozygosities (H_E and H_O) and the deviations from the Hardy-Weinberg equilibrium (dev HWE) of the eastern and western Slovak Imperial Eagle subpopulations (ns=not significant; * P<0.05; ** P<0.01; *** P<0.001)
Tab. 3. Očakávaná a pozorovaná heterozygotnosť (H_E a H_O) a odchýlky od Hardy-Weinbergovej rovnováhy (dev HWE) u východoslovenskej a západoslovenskej subpopulácie orla kráľovského (ns=štatisticky nevýznamné; * P<0.05; ** P<0.01; *** P<0.001)

locus / lokus	east / východ (n = 21)						west / západ (n = 22)					
	HO	HE	dev HWE				HO	HE	dev HWE			
			DF	ChiSq	prob	signif			DF	ChiSq	prob	signif
G12	0.48	0.49	1.00	0.02	0.90	ns	0.41	0.49	1.00	0.61	0.44	ns
G04	0.33	0.30	6.00	0.84	0.99	ns	0.41	0.47	10.00	45.85	0.00	***
G11	0.38	0.31	1.00	1.16	0.28	ns	0.45	0.40	1.00	0.47	0.49	ns
G13	1.00	0.57	3.00	6.00	0.11	ns	1.00	0.59	3.00	4.00	0.26	ns
G15	0.62	0.70	6.00	4.36	0.63	ns	0.85	0.72	15.00	28.37	0.02	*
G14	0.57	0.49	1.00	0.58	0.45	ns	0.55	0.48	1.00	0.36	0.55	ns
G09	0.35	0.53	3.00	12.45	0.01	**	0.33	0.41	3.00	3.61	0.31	ns
Aa02	0.95	0.77	15.00	19.59	0.19	ns	0.90	0.74	15.00	19.74	0.18	ns
Aa39	1.00	0.84	28.00	45.50	0.02	*	1.00	0.81	21.00	14.76	0.84	ns
Aa35	1.00	0.76	15.00	15.74	0.40	ns	1.00	0.79	10.00	6.09	0.81	ns

Genetic structure in the Slovak population

Based on the mtDNA CR haplotypes, genetic diversity was similar in the eastern (h = 0.74, π = 0.0047) and western (h = 0.71, π = 0.0055) subpopulation. On the other hand, CR haplotypes showed an unequal distribution, since L and S haplotypes were present only in the eastern, G and D only in the western region (L, S). Shared haplotypes were similarly distributed in the eastern and in the western subpopulation (Fig. 1.).

Genetic structure analysis of the mtDNA CR haplotypes showed a marginally significant genetic separation between the eastern and western subpopulations using both AMOVA (Fst = 0.064, P = 0.08) and nonparametric exact test (P = 0.04). Similarly, AMOVA analysis of microsatellite data resulted in a marginally significant genetic separation between the eastern and western breeding area (Fst = 0.015, P = 0.05).

Discussion

According to our results, both sampling methods (shed feathers and blood samples collected on special impregnated paper) can provide appropriate DNA samples for genetic examinations without high costs and can be easily used by field ornithologists. We believe that the low efficiency found by the feather samples can most likely be explained by the different storage conditions, since samples collected in other countries with similar size and age yielded appropriate results in almost all cases (Vili unpublished data). It is important to store the feathers in a dark, dry and cool place, to separate the blood clots as soon as possible and store these samples in a freezer (-20°C) until DNA-extraction to avoid DNA degradation caused by ultraviolet radiation, heat and humidity (Thacker et al. 2006).

The sex ratio differences in adult birds' feather samples can be explained by the phenomenon that males are moulting usually in their roosting sites, while females mostly sit on the nests where feathers are more likely to be found. The molecular method used for adult sexing was reliable and can be easily applied to other species as well (Fridolfsson & Ellegren 1999).

Similarly to the results of Rudnick et al. (2005) we found no significant difference in the sex ratio among chicks. We also found that sexing of the chicks based on morphological features can result inappropriate result in one-third of the cases, which enhances the importance of genetic methods to get correct data on the sex ratio of chicks.

The significant deviation from the Hardy-Weinberg equilibrium found in four loci (East: loci G09 and Aa39, West: loci G04 and G15) could be due to the large migration capacity of the species, however the relatively low sample size (N_{East} = 21, N_{West} = 22) could also affect the results.

Considering the size of the population the PI value of our microsatellite marker set was low enough to use these loci for individual identification (Taberlet & Luikart 1999). Since DNA fingerprinting is reliable, data could be integrated in a common Hungarian-Slovak "DNA-

Tab. 4. mtDNA control region, haplotypes found in Slovak Imperial Eagles, along with the variable positions and their distribution in the two breeding regions (ES – East Slovakia, WS – West Slovakia). Haplotype D, E and G were originally described by Martínez-Cruz et al. (2004, EMBL accession numbers: AJ567367, AJ57880- AJ57882 and AJ57884)

Tab. 4. Kontrolný región mtDNA a haplotypy zistené u orlov kráľovských na Slovensku s odlišnými pozíciami a ich distribúciou v dvoch hniezdnych regiónoch (ES – východné Slovensko, WS – západné Slovensko). Haplotypy D, E a G pôvodne opísali Martínez-Cruz et al. (2004, EMBL prístupové čísla: AJ567367, AJ57880- AJ57882 a AJ57884)

haplotype / haplotyp	nucleotide position / pozícia nukleotidov	region / oblasť	
	0 0 1 1 2 2 2		
	4 9 1 3 1 2 3	ES	WS
	5 6 8 4 9 7 9		
D	A A C G T A T	-	2
E	. . . . C . .	5	6
G	. G T . C G .	-	3
K	G G T . C G .	2	1
L	. . . . C G .	4	-
S	. . . A C . .	1	-

-fingerprint" database, using them for subsequent individual identification of the same specimens if resampled (i.e. moulted feathers or a carcass found at breeding or temporary settlement areas).

Distribution of microsatellite allele frequencies and mtDNA control region haplotypes suggest a small genetic difference between Imperial Eagle subpopulations in East and West Slovakia. Our results show that, in spite of the large dispersal capacity of the species, a relatively small geographic distance can also decrease the exchange rate of individuals between subpopulations, which could be explained by the relatively high natal site fidelity of the species. On the other hand the difference we found is slight, which is similar to the results of Martínez-Cruz et al. (2004), who found no significant difference between populations of the Spanish Imperial Eagle with similar or slightly greater distances.

Our results strengthen the previous hypothesis that we should handle the Carpathian Basin's breeding birds in one uniform population (Horváth et al. 2002), although the western and eastern breeding nuclei can be handled as subpopulations, with slightly limited gene-flow among them. Further studies with similar methodology need to involve larger sample sizes from the southern Hungarian subpopulations; to reveal more precisely the genetic structure and gene flow patterns between subpopulations of the Imperial Eagle in the entire Carpathian Basin.

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