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Multiplex PCR amplification of 13 microsatellite loci for *Aquila chrysaetos* in forensic applications

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Abstract: The golden eagle (*Aquila chrysaetos*) is an endangered raptor, which is threatened mainly by illegal egg and nestling robbery. Here we describe a fluorescently labeled, multiplex PCR method using 13 microsatellite markers, which provides a powerful tool for the individual identification and parentage testing of the Golden eagle. This test should be applicable to both forensic analysis and population studies. Fifteen polymorphic loci from *A. chrysaetos* were cross-amplified. Subsequent PCR condition optimization led to the successful co-amplification of 13 different loci in a single PCR reaction. Fifty samples from wild-living individuals and 89 samples from captive-bred individuals were examined. The results indicated that both populations have similar levels of moderate inbreeding, unsurprising in a small population. This probability of excluding a random individual in parentage analysis was 0.9912 for the wild population and 0.9932 in the captive-bred one in the case that both the individual and its mother were examined together. The probability of identity was estimated to be 3×10^{-8} for the wild and 4×10^{-8} for the captive-bred populations. Given the size of the Slovak golden eagle population, this test should therefore be sufficient to reliably identify individual raptors and assess parentage in both conservation studies and forensic analysis.

Key words: *Aquila chrysaetos*; golden eagle; microsatellites; multiplex-PCR; parentage assessment.

Abbreviations: H_E, expected heterozygosity, H_O, observed heterozygosity, HWE, Hardy-Weinberg equilibrium; P_{E1} and P_{E2}, paternity exclusion powers; PIC, polymorphic information content; P_{ID}, probability of identity; STR, short tandem repeat.

Introduction

The golden eagle (*Aquila chrysaetos*), once one of the most common raptors, is primarily distributed across the European mild climate zone, North Asia, North America, North Africa and Japan. In the last two centuries, it became critically endangered and at risk of extinction in several European countries. Its European population size was substantially reduced during the 19th century, primarily by hunting, and was close to extinction by the beginning of the 20th century. At present, its occurrence in Europe is mostly restricted to the alpine zone in the Alps (Suchentrunk et al. 1999) and the High and Low Tatras (Danko et al. 2002). The IUCN red list indicates that the golden eagle is completely extinct in Ireland, and, based on the Carpahtian List of Endangered Species (Witkowski et al. 2003), extinct in the wild in the Czech Republic and critically endangered in Poland, Slovakia, Ukraine and vulnerable in Hungary and Romania. These circumstances have led to the legal protection of the golden eagle in the Czech Republic, Hungary, Poland, Romania, Ukraine, and Slovakia as well as several re-introduction efforts, most recently in Ireland (O'Toole et al. 2002) and in the Czech Republic. As a result of these and other conservation efforts, the number of golden eagles in many parts of Europe has begun to increase, but it is still listed as a rare species in Europe.

Endangered species have only a small population and, consequently, may have reduced genetic variation as a result of inbreeding which, in turn, might limit the ability of the species to adapt to environmental changes (Lande & Barrowclough 1987; Reed & Frankham 2003). A severe decrease in population size may also lead to an increase in the frequency of rare, deleterious recessive alleles, which would increase the risk of population extinction (Frankham 1998; Brook et al. 2002; O'Grady et al. 2006). Consequently, the study of the genetic structure of an endangered species may be quite important for conservation efforts (Bayle 1999; Suchentrunk et al. 1999; Pedrini & Sergio 2001; Hille et al. 2003; Bourke et al 2010).

In addition, one of the largest threats to the wild population in Slovakia is egg and nestling robbery. Consequently, Slovak legislation requires paternity testing for all captive-bred individuals to show that they are

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offspring of legally registered parents. In this environment, reliable methods for parentage testing are required for both conservation and wildlife forensic analysis. The multilocus DNA fingerprint method previously used for parentage testing (Rychlik et al. 1994), like all multilocus DNA fingerprint tests, suffers from technical, theoretical, and statistical difficulties that makes it difficult to use or inappropriate in studies of complex mating systems and parentage assessments (Read 2006). The analysis of a large number of samples can be expensive and labor-intensive, and scoring of a fingerprint gel is time-consuming. Furthermore the relative migration of non-adjacent fragments can be difficult to compare and comparisons between samples on different gels are impossible. The technique requires a large amount of high-quality genomic DNA and repeatability can be poor (Bruford et al. 1998). Finally, McRae & Amos (1999) make the valuable point that, when incest has occurred, as is possible in a small population, multilocus minisatellite DNA fingerprinting has limited ability to resolve parentage. Our goal was to design a reliable and powerful DNA-based identification method in order to characterize the genotype of both wild eagles and those living in captivity in order to obtain allele frequencies to characterize their gene pool variability. We also hoped to determine if inbreeding in wild-living eagles was occurring based on decreasing heterozygosity in the wild population.

In recent years, a number of microsatellite techniques have been developed for several raptor species (Nesje & Roed 2000; Nesje et al. 2000; Martínez-Cruz 2002; Busch et al. 2005; Ortego et al. 2007; Tingay et al. 2007; Padilla et al. 2008) and a multiplex PCR assay has also been established for the white-tailed sea eagle (Hailer et al. 2005). Multiplex PCR methods reduce several analyses into one (Edwards & Gibbs 1994), thereby simplifying analysis. When combined with knowledge of highly informative short tandem repeat (STR) polymorphisms and modern molecular biology techniques (e.g. laser-based detection of fluorescently-labeled PCR products), it can be used to create an exact, reproducible, and high-throughput DNA-based identification method (see, for example, Bonnet et al. 2002). Here, we extend these previously established techniques to the golden eagle and report the results of parentage assessments for 50 wild-living and 89 captive-bred individuals.

Material and methods

Blood samples and DNA purification

Total genomic DNA was extracted from blood with or without anticoagulant using standard phenol-chloroform extraction or silica columns (Qiamp Blood & Tissue Kit, Qiagen). Nonidet P-40 (Roche) treatment of all blood samples without anticoagulant was applied prior to purification. Altogether 139 samples were tested: 50 from wild-living and 89 from captive-bred golden eagles. All samples from wild-living birds were collected throughout two years from nestlings by members of the Slovak Environmental Inspection and the Slovak Environmental Agency during a ten-year Golden Eagle Monitoring Project (1994–2003). Samples were taken from nests in the wild when the nestlings were approximately 45 days old and were ringbarking. Long-term monitoring of these nests ensured that each sample used in this study originated from only one nestling belonging to one breeding pair; this avoids inadvertently comparing closely related individuals. Samples from captive raptors were collected either by their keepers or by veterinarians. From this set, we used only samples that had come from unrelated individuals. In addition, we also analyzed 15 related individuals (full siblings) from three breeding pairs, and 15 half siblings to investigate whether each of them has unique genotype. These related individuals were not included in the data set used for estimating heterozygosity, allele frequencies, etc.

Analyzed markers

For cross-amplification, 15 polymorphic markers were tested in the golden eagle population for amplification and genetic variability in a manner similar to that previously described by Nesje & Roed (2000) and Martínez-Cruz et al. (2002). These loci were chosen from the original studies in a manner according to the highest observed heterozygosities; however, due to either low or no variability in the population under study, two loci (NVH fr144-2 and NVHfr190) were excluded from the initial panel. Prior to multiplexing each locus was analyzed and standardized separately. Several primer concentration ratios were tested when multiplexing to obtain a sufficient amplification yield for all loci. Finally, primers specific to 13 polymorphic autosomal loci (NVH fr142, NVH fr144-2, NVH fr190, NVH fr206, Aa02, Aa04, Aa11, Aa15, Aa26, Aa27, Aa35, Aa36, Aa39, Aa43, and Aa50) were multiplexed into one PCR reaction. Primers that corresponded to loci, which overlapped in PCR product size, were labeled with different fluorescent dyes.

PCR amplification and fragment analysis

The PCR reaction was carried out in a 20 μL final reaction volume containing 50 ng genomic DNA, 0.125–0.375 μM primers, 3 mM $MgCl_2$, and 0.2 mM dNTPs. Two units of Taq polymerase per reaction and 2× PCR buffer to a final concentration of 150 mM Tris-HCl, 40 mM $(NH_4)_2SO_4$, and 0.02% Tween-20 was added. Primers specific to the Aa02, Aa04, Aa11, Aa15, Aa26, Aa27, Aa35, Aa36, Aa39, Aa43, Aa50, NVHfr142, and NVHfr206 loci were added in the following concentration ratio: 2:2:2:1:1:1:2:2:3:1:1:1:1. Table 1 shows details of the primer sequences, concentrations and applied fluorescent labels. The cycling conditions, using a T1 Thermocycler (Biometra), were set as follows: 6 min and 94 °C, followed by 35 cycles of 40 s at 94 °C, 60 s at 46 °C and 40 s at 68 °C, and a final cycle of 15 min at 68 °C. PCR amplicons were analyzed using an ABI 3100Avant genetic analyzer using a standard fragment analysis protocol with the GeneScan-500 LIZ Size Standard (Applied Biosystems) and the GeneMapper 3.5 software for allele scoring.

Statistics

Based on the observed genotypes of 50 unrelated wild individuals, the genetic diversity of the wild population was estimated. Allele frequencies, the Polymorphic information content (PIC), the expected (H_E) and observed (H_O) heterozygosity and paternity exclusion powers P_{E1} and P_{E2} were calculated using CERVUS 2.0 (Slate et al. 2000a). Probability of identity (P_{ID}) was estimated with GIMLET (Valiére 2002) using an equation from Kendall & Stewart (1977). The probability of identity within relative individuals (P_{IDsib}) was estimated according to Waits et al. (2001).

Table 1. Characterization of 13 microsatellite loci used for individual identification of *Aquila chrysaetos*, showing primer sequences, primer concentration and dye labels for multiplex PCR, and the observed diversity in samples from 50 wild-living and 89 captive-bred individuals.

Locus	Primer sequence	Primer (μM)	Dye	Wild-living		Captive-bred	
				No. of alleles	Size	No. of alleles	Size
Aa02	CTGCAGATTTCACCTGTTCTG CTTCCAGGTCTTGCAGTTTACC	0.250	VIC	4	133–139	5	133–141
Aa04	TGCAGCTCAAAAGCAAAGG CAACCCCAACTCTCACACCT	0.250	NED	7	121–151	10	123–155
Aa11	ACGAGCTTATCTTTGACCAAGC CTTTGTTTCAGCTGTTCCAGG	0.250	VIC	3	257–269	7	245–267
Aa15	TCACTGACCTGCCCTCTACA CCAACCCTCTAGTCGTCCAC	0.125	FAM	3	198–206	5	198–206
Aa26	GCAAAGGTAAACTGCATCTGG ATGCACTATTGGTAAACAGGCA	0.125	PET	8	140–154	9	138–154
Aa27	GAGATGTCTTCACAGCTTGGC AAGTCTCAGAGACTGACGGACC	0.125	VIC	4	84–96	3	84–94
Aa35	GCAGCAGAAAGTGCATACGA GACCAAATGAAATGCGCC	0.250	FAM	4	229–255	7	229–257
Aa36	ACAGGCCAGCACCAAGAG TTTGGAGCCATTGTTACCGT	0.250	PET	5	96–104	8	84–104
Aa39	TTCTGTTTTTCCACTTGCTTG TATTGAGCTCACAAAAACAAAGG	0.375	VIC	7	185–209	9	185–209
Aa43	CCACACTGAGAAACTCCTGTTG TTCCTGAGAGCTCTTCCTGC	0.125	FAM	5	105–129	5	105–113
Aa50	AACATGGCAATGTGTTTCGA ATTGACGCTGCAAACAGATG	0.125	PET	2	211–217	4	209–219
NVHfr142	CCACCCCTCTGCCACTCA CCCCTGTCAGCTAAACACATCAC	0.125	PET	6	179–191	9	173–191
NVHfr206	ATCTAATGGGCTTTCCTGGATTT GACATTTTCCTCATAGGCAACTGA	0.125	FAM	2	159–161	2	159–161

Deviations from Hardy-Weinberg equilibrium (HWE) for each locus from each group of individuals (wild-living and captive-bred) were computed using the web-based version of GENEPOP 3.4 (Raymond & Rousset 1995). GENEPOP 3.4 was also used to assess genetic differentiation by comparing heterozygosities and allele frequencies (F-statistics). These F-statistics (F_{IS}, F_{ST}, F_{IT}) were estimated using a method proposed by Weir & Cockerham (1984). F_{ST} estimates the variation produced by differences between populations, F_{IS} estimates the variation inside populations, and F_{IT} is the inbreeding coefficient of one individual relative to the total population.

Results and discussion

The successful co-amplification of 13 different STR polymorphisms after optimization by single multiplex PCR is shown in Figure 1; the genotype profiles are from three unrelated individuals. The allelic diversity of these 13 microsatellite markers in the sample of 50 wild-living eagles ranged from 2 to 8 with an average allelic diversity across loci of 4.62 per locus with a mean expected heterozygosity of 0.489 and probability of identity $P_{ID} = 3 \times 10^{-8}$ (Table 2). Table 3 shows more details about the observed alleles, their frequencies and PCR product sizes. A slightly higher allelic diversity was observed in the group of captive-bred individuals, which ranged from 2 to 10 with a mean number of alleles per locus of 6.38. This probably results from the intentional mating of individuals from distant regions (the Caucasus or Scandinavia) to avoid inbreeding. The average PIC, which expresses the informative value of the polymorphic STR markers, estimated by CERVUS, was relatively low: 0.447 for wild-living eagles and 0.441 for birds bred in captivity. The probability that a random father could be excluded when only an offspring is analyzed in a paternity test is rather high (P_{E1} 0.8977 for the wild and 0.9150 for the captive populations) and it is even higher if both the offspring and the mother are analyzed together (P_{E2} 0.9912 and 0.9932 for the wild and captive populations, respectively). This means that we are able to state whether a given individual is, in fact, the offspring of its alleged parents with 99.12 % or 99.32 % probability.

Some breeders interbred Slovak golden eagles with individuals from Caucasian or Nordic countries, which introduced new alleles that are not present in the wild-living Golden eagles in Slovakia. These unique alleles are indicated boldface in Table 3, along with their observed frequencies. Additionally, some alleles were found only in the wild population, but not in the captive one, including locus Aa04 alleles 121, 149; locus Aa11 allele 269, locus Aa27 allele 96; locus Aa43 alleles 127 and 129. The true origin of all birds bred in captivity, in particular the first breeding pairs, is not known, but either some or all of them originated from the wild Slovak population. Because it is possible that some breeders are mating relatives and because the population of wild golden eagles is small, we estimated the decline of heterozygosity in individuals, which would indicate inbreeding, and evaluated the genetic differentiation between these two groups.

GENEPOP was used to determine deviations from

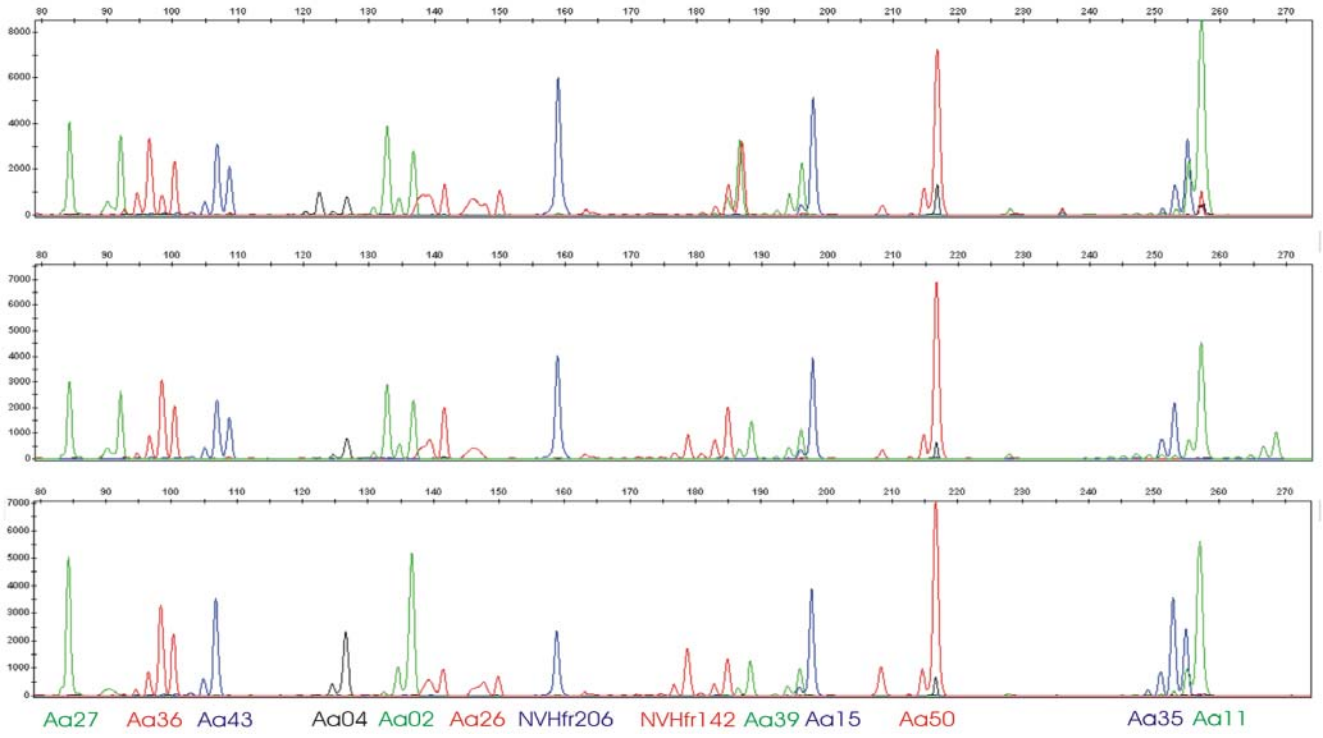

Fig. 1. Electrophoretogram showing the genotypes of three individuals from 13 autosomal STR polymorphisms analyzed by multiplex PCR and capillary fragment analysis. This overlay shows four different fluorescent dyes labeling loci Aa45, Aa35, Aa15, and NVHfr206 with FAM, loci Aa27, Aa02, Aa39, and Aa11 with VIC, Aa04 with NED and Aa36, Aa26, Aa50 and NVHfr142 with PET.

Table 2. Population characteristics for 13 STR polymorphisms genotyped in wild-living ($n = 50$) and captive-bred ($n = 89$) *Aquila chrysaetos*. An exact test of the HWE showed a significant heterozygote deficit in some loci ($^*P < 0.001$).

Locus	Wild-living						Capture-bred					
	H_O	H_E	PIC	P_{E1}	P_{E2}	F_{IS}	H_O	H_E	PIC	P_{E1}	P_{E2}	F_{IS}
Aa02	0.380	0.421	0.378	0.089	0.220	+0.098	0.425	0.445	0.415	0.041	0.154	+0.044
Aa04	0.196*	0.470	0.443	0.122	0.284	+0.586	0.333*	0.593	0.534	0.191	0.346	+0.439
Aa11	0.204	0.187	0.171	0.017	0.088	−0.092	0.167	0.212	0.205	0.023	0.115	+0.213
Aa15	0.520	0.444	0.395	0.096	0.228	−0.174	0.302	0.356	0.328	0.064	0.188	+0.151
Aa26	0.773	0.670	0.602	0.248	0.405	−0.154	0.597*	0.774	0.732	0.378	0.555	+0.230
Aa27	0.429	0.498	0.455	0.128	0.281	+0.163	0.679	0.588	0.517	0.171	0.313	−0.143
Aa35	0.620	0.678	0.602	0.233	0.386	+0.086	0.547*	0.619	0.554	0.205	0.357	+0.117
Aa36	0.300*	0.477	0.448	0.123	0.284	+0.373	0.318	0.368	0.352	0.073	0.215	+0.135
Aa39	0.820	0.771	0.733	0.380	0.562	−0.064	0.713	0.755	0.718	0.364	0.545	+0.056
Aa43	0.260*	0.504	0.451	0.128	0.272	+0.486	0.270	0.283	0.269	0.041	0.154	+0.047
Aa50	0.160	0.149	0.136	0.011	0.068	−0.077	0.270	0.260	0.241	0.034	0.131	−0.037
NVHfr142	0.620*	0.761	0.716	0.354	0.534	+0.187	0.736	0.798*	0.765	0.423	0.602	+0.079
NVHfr206	0.375	0.333	0.275	0.054	0.138	−0.126	0.069	0.109	0.102	0.006	0.051	+0.368

the HWE and for estimating the F-statistics. These results are summarized in Table 2. F_{IS} is an estimate of the genetic variation within a population and can be used to infer the level of heterozygote deficiency or excess in the analyzed individuals (Weir & Cockerham 1984; Weir & Hill 2002). Loci Aa04, Aa27, Aa36, Aa43 and NVHfr142 in the wild population and loci Aa04, Aa11, Aa15, Aa26, Aa35, Aa36 and NVHfr206 in the captive one showed values significantly greater than zero, which indicates an excess of homozygotes, possibly arising from inbreeding. Loci Aa15 and NVHfr206 in wild and locus Aa27 in captive birds, in contrast, had negative values, indicating an excess of heterozygotes. Loci Aa39 and Aa11 in the wild population and Aa50 in both populations showed a small excess of heterozygotes. Over all loci in both populations, F_{IS} was +0.1142, showing a moderate average excess of homozygotes. This moderate excess of homozygotes in both populations indicates that inbreeding has most likely occurred in these populations because inbreeding has been shown to be the most likely cause of heterozygote deficiencies (Genlous & Björn 2003).

F_{ST}, an estimate of the genetic difference between the wild and captive populations, was 0.0196, which indicates that the level of genetic divergence between these populations is quite low. This result was expected

Table 3. Observed allele frequencies for 13 autosomal STR polymorphisms from *Aquila chrysaetos* wild-living ($n = 50$) and captive-bred ($n = 89$) individuals, along with number of heterozygotes (Hets) and homozygotes (Homs). Alleles unique to either the wild or captive populations are shown in bold.

Locus	Wild-living				Captive-bred			
	Size bp	Hets	Homs	Frequency	Size bp	Hets	Homs	Frequency
Aa02	133	4	0	0.0400	133	10	0	0.0575
	135	14	2	0.1800	135	18	3	0.1379
	137	16	29	0.7400	137	33	47	0.7299
	139	4	0	0.400	139	8	0	0.0460
					141	5	0	0.0287
Aa04	**121**	0	1	0.0217	123	14	11	0.2727
	123	5	3	0.1196	127	20	28	0.5758
	127	8	29	0.7174	**129**	0	2	0.0303
	141	0	1	0.0217	**135**	1	0	0.0076
	147	1	2	0.0543	**139**	1	0	0.0076
	149	2	0	0.0217	141	1	0	0.0076
	151	2	1	0.0435	**143**	1	0	0.0076
					147	0	1	0.0152
					151	5	2	0.0682
					155	1	0	0.0076
Aa11	257	10	39	0.8980	**245**	2	1	0.0238
	265	9	0	0.0918	**255**	1	0	0.0060
	269	1	0	0.0102	257	11	69	0.8869
					259	2	0	0.0119
					263	1	0	0.0060
					265	7	0	0.0417
					267	4	0	0.0238
Aa15	198	24	24	0.7200	198	24	56	0.7907
	204	10	0	0.1000	**200**	2	0	0.0116
	206	18	0	0.1800	**202**	2	0	0.0116
					204	9	0	0.0523
					206	15	4	0.1337
Aa26	140	1	0	0.0114	**138**	1	0	0.0069
	142	26	5	0.4091	140	1	1	0.0208
	144	1	0	0.0114	142	24	7	0.2639
	146	1	0	0.0114	144	2	0	0.0139
	148	1	0	0.0114	146	5	1	0.0486
	150	27	4	0.3977	148	3	1	0.0347
	152	3	1	0.0568	150	32	4	0.2778
	154	8	0	0.0909	152	10	15	0.2778
					154	8	0	0.0556
Aa27	84	17	25	0.6837	84	53	19	0.5617
	92	8	1	0.1020	92	20	4	0.1728
	94	12	2	0.1633	94	37	3	0.2654
	96	5	0	0.0510				
Aa35	229	14	6	0.2600	229	13	3	0.1105
	231	2	0	0.0200	231	0	3	0.0349
	253	23	8	0.3900	**249**	2	0	0.0116
	255	23	5	0.3300	**251**	1	0	0.0058
					253	43	24	0.5291
					255	34	9	0.3023
					257	1	0	0.0058
Aa36	96	5	2	0.0900	**84**	2	0	0.0114
	98	3	0	0.0300	**86**	2	0	0.0114
	100	9	31	0.7100	**88**	1	0	0.0057
	102	7	0	0.0700	96	13	1	0.0852
	104	6	2	0.1000	98	6	0	0.0341
					100	27	56	0.7898
					102	1	3	0.0398
					104	4	0	0.0227
Aa39	185	13	1	0.1500	185	16	1	0.1034
	187	16	2	0.2000	187	30	6	0.2414
	189	27	6	0.3900	189	40	15	0.4023
	193	12	0	0.1200	**191**	2	0	0.0115
	195	2	0	0.0200	193	20	0	0.1149
	197	7	0	0.0700	195	8	1	0.0575
	209	5	0	0.0500	197	6	1	0.0460
					201	0	1	0.0115
					209	2	0	0.0115

Table 3. (continued)

Locus	Wild-living				Captive-bred			
	Size bp	Hets	Homs	Frequency	Size bp	Hets	Homs	Frequency
Aa43	105	7	2	0.1100	105	6	2	0.0562
	107	9	29	0.6700	107	24	63	0.8427
	109	8	6	0.2000	109	12	0	0.0674
	127	1	0	0.0100	**111**	5	0	0.0281
	129	1	0	0.0100	**113**	1	0	0.0056
Aa50	211	8	0	0.0800	**209**	3	0	0.0169
	217	8	42	0.9200	211	17	1	0.1067
					217	24	64	0.8539
					219	4	0	0.0225
NVHfr142	179	9	0	0.0900	**173**	2	0	0.0115
	183	11	0	0.1100	**177**	0	1	0.0115
	185	21	8	0.3700	179	26	1	0.1609
	187	11	6	0.2300	**181**	5	0	0.0287
	189	1	0	0.0100	183	17	5	0.1552
	191	9	5	0.1900	185	41	8	0.3276
					187	21	7	0.2011
					189	3	0	0.172
					191	13	1	0.0862
NVHfr206	159	18	29	0.7917	159	6	79	0.9425
	161	18	1	0.2083	161	6	2	0.0575

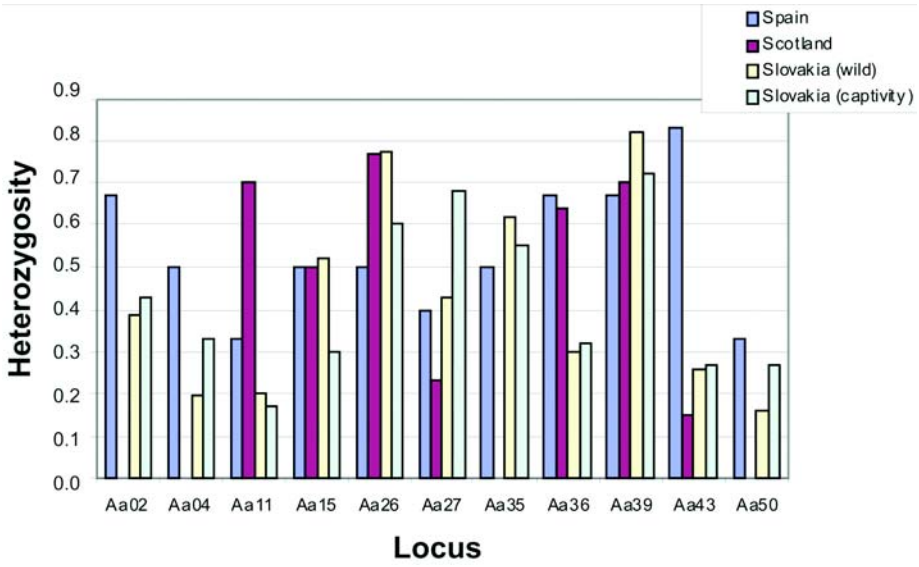

Fig. 2. Comparison of observed heterozygosity (H_O) of particular loci in Slovak wild-living eagles, Slovak birds bred in captivity and individuals analyzed in Spain (Martinez-Cruz et al. 2002) and Scotland (Bourke & Dawson 2006).

because the original breeding pairs were most likely taken from the wild population of Slovakia or nearby regions. Both F_{IS} and F_{ST} indicate that the level of inbreeding in the captive population is approximately the same as that in the wild one.

A graphical comparison of the observed heterozygosity (H_O) of particular loci in Slovak wild-living eagles, Slovak birds bred in captivity, and individuals analyzed in Spain (Martínez-Cruz et al. 2002) and Scotland (Bourke & Dawson 2006) is shown in Figure 2. A significantly higher heterozygosity was observed for locus Aa11 in the Scottish population (0.70) while in the Spanish one it was only 0.33 and in the Slovak wild and captive populations, it was 0.2 and 0.17, respectively. A similar situation was also seen for loci Aa15, Aa26 and Aa39. Furthermore, the observed heterozygosity for locus Aa36 in the Slovak populations (0.30 wild and 0.32 captive) reached only half of that for the Spanish (0.67) or Scottish (0.64) populations. On the other hand, a significantly higher heterozygosity was observed in the Spanish population for locus Aa43 (0.83) but not in either the Scottish (0.15) or Slovak populations (0.26 wild and 0.27 captive). From this, it would appear that the Slovak populations are the most inbred.

It is presently debated, however, whether and to what extent heterozygosity reflects the actual level of inbreeding in a real population (Balloux et al. 2004). Many recent studies report that individual heterozygosity, estimated by microsatellite markers, is correlated with key aspects of individual fitness, such as survival

(Coulson et al. 1999), disease resistance (Coltman et al. 1999), fecundity (Amos et al. 2001) and lifetime breeding success (Slate et al. 2000b); it is also correlated with features involved in mate selection, such as birdsong complexity (Marshall et al. 2003). Studies reporting negative results, on the other hand, seem rare. Brook et al. (2002) provided a strong evidence that inbreeding depression elevates extinction risk in most out-breeding threatened species.

Given the level of inbreeding that these results imply, it is essential for the reliability of this method that it should also be able to determine parentage in the case of relatives, for example between full siblings. To verify the applicability of this method to parentage assessment in captivity bred birds, the 13 microsatellite loci were also analyzed in 30 related individuals (15 full siblings, 15 half siblings). In no case two individuals with the same genotype were detected; no two individuals had identical alleles in all analyzed loci. These results indicate that the proposed series of 13 STR loci can be used to assess parentage in either wild-living or captive-bred individuals.

Owing to widespread nestling robbery, Slovak legislation requires paternity testing for all captive-bred individuals to show that they are offspring of legally registered parents. Such a test requires high discrimination power, reproducibility and reliability. The multiplex PCR test described here provides a rapid and reliable method for unambiguously determining if a given raptor is actually the progeny of its alleged parents. Using the program GIMLET the method shows that the unbiased probability of identity (P_{ID}unbias) for the combination of all 13 STR loci examined in this study was $P_{\mathrm{ID}}\mathrm{unbias} = 3 \times 10^{-8}$ for wild-living population and $4{\times}10^{-8}$ for birds bred in captivity, while the probability of identity between related individuals was $P_{\mathrm{ID}}\mathrm{sibs} = 0.0008$ for the wild and 0.0009 for the captive populations. This means that we can expect one of 1,250 individuals from the wild population or one of 1,111 individuals from the captive one to share the same genotype with an adventitiously chosen individual from its relatives. These values should be sufficient to distinguish individual raptors because the population of wild-living golden eagles in Slovakia is estimated to be only 70–90 pairs, i.e. 140–180 individuals. Taylor et al. (1994) considered a P_{ID} of 0.01–0.001 (1 of 100 to 1 of 1,000 individuals) to be acceptable for forensic investigations of the *Lasiorhinus krefftii* population because the remaining population has fewer than 100 individuals. If it becomes necessary, however, the informative value of PIC, P_{E1} and P_{E2} can be increased by including more STR polymorphic markers, as published by Busch et al. (2005), Hailer et al. (2005) and Tingay et al. (2007) for the genera *Aquila* and *Haliaeetus*.

In summary, we have developed a multiplex PCR assay, which is able to correctly assess parentage in the golden eagle with a certainty of 99.12% from the wild and 99.32% from the captive populations. Applying this test to 50 unrelated wild-living individuals and 89 unrelated captive-bred ones a moderate excess of homozygotes in both populations was revealed, indicating some inbreeding in both populations. This test should be useful not only for conservation management, but also for forensic analysis, in the case that there are some doubts as to if a particular individual is actually the offspring of legally registered parents.

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