Eighteen polymorphic microsatellite markers for the highly endangered Spanish imperial eagle (*Aquila adalberti*) and related species

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Abstract

Here we describe the development of 18 polymorphic microsatellite markers for the endangered Spanish imperial eagle (*Aquila adalberti*). Microsatellites were tested in five other raptor species. These markers were revealed as good molecular tools for genetic population studies, individual identification and parentage assessment in Spanish imperial eagle and closely related species.

Keywords: *Aquila adalberti*, individual identification, microsatellites, paternity assessment, population genetics, Spanish imperial eagle

The Spanish imperial eagle (*Aquila adalberti*) is one of the most endangered raptors in the world (classified in the CITES Appendix I). During the 20th century, the species has suffered a strong demographic bottleneck (González *et al*. 1989). Currently, around 131 breeding pairs (year-2000 census by SEO Bird Life) are distributed into several breeding nuclei in southwest Spain (Ferrer 1993). The present situation of the species remains critical mainly because of human pressures and the sharp decline of rabbit populations, which are their main prey. The development of molecular genetic tools is deemed crucial for the management and conservation of this species.

Microsatellites are particulary suitable for studying endangered species as they allow working with samples collected noninvasively, such as feathers and eggshells (Pearce *et al*. 1997; Taberlet *et al*. 1999; Strausberger & Ashley 2001). To our knowledge, no microsatellite markers have been described for any *Aquila* species and they have only been developed for three other raptor species (Nesje & Roed 2000a; Gautschi *et al*. 2000; Nesje *et al*. 2000b). Here we characterize 18 polymorphic microsatellite markers developed for the Spanish imperial eagle. They will allow the assessment of the genetic structure and variability of remaining populations (Nesje *et al*. 2000c).

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Microsatellites were isolated from a GT dinucleotide repeat enriched genomic library using a modified nonradioactive capture-hybridization method (Refseth *et al*. 1997) modified in Sarno *et al*. (2000). Genomic DNA was extracted from a blood sample of a female chick Spanish imperial eagle sampled in Aceuche (Cáceres) in 1996, using a standard phenol-cloroform protocol (Sambrook *et al*. 1989). Microsatellite isolation procedure was as described in Sarno *et al*. (2000).

Nonredundant clones were selected from fragments that contained only one uninterrupted microsatellite composed of at least 10 tandem repeats and that had flanking sequences that were long enough to design primers. Forty-four primer pairs were designed from selected clones using the Microsatellite Target Identification Program (R. Stevens and V.A. David, unpublished) and the program primer 3 (available at www.genome.wi.mit.edu/cgibin/primer/primer3_www.cgi). From an initial amplification in 15 unrelated individuals using dUTP labelling (David & Menotti-Raymond 1998), 17 polymorphic dinucleotide and one tetranucleotide were selected. Forward fluorescently labelled markers were tested further in a total of 38 Spanish imperial eagles and in other raptor species. Polymerase chain reactions (PCRs) were carried out in a MJ Research PTC-100 thermocycler in 20 μL containing 16 mm $(NH4)SO4$, 2.5 mm $MgCl_2$, 0.25 mm of each dNTP, 0.5 U Taq DNA polymerase (Bioline), 0.25 μm of each primer and 62.5 ng DNA, under the following conditions: an initial denaturation

Table 1 Characterization of 18 A adalberti microsatellite loci in 38 individuals from several breeding nuclei

Locus	Genbank accession numbers	Primer sequences 5′–3′	Repeat motif	Size range (bp)	Number of alleles	H_O	H_E	HWE	
								P-value	SE
Aa02	AF469500	F^: CTGCAGATTTCACCTGTTCTG R: CTTCCAGGTCTTGCAGTTTACC	$(GT)_{19}$	141–155	4	0.45	0.41	0.9046	0.0034
Aa04	AF469506	F^: TGCAGCTCAAAAGCAAAGG R: CAACCCCAACTCTCACACCT	$(GT)_{12}$	126–130	3	0.61	0.64	0.1025	0.0032
Aa11	AF469497	F^: ACGAGCTTATCTTTGACCAAGC R: CTTTGTTTCAGCTGTTCCAGG	$(CA)_{11}$	246–252	3	0.34	0.29	0.6263	0.0051
Aa12	AF469498	F^: TCATCAACCTGACCCTTTCC R: TGCACTGAAGTTTCTCGGC	$(GT)_{12}$	133–135	2	0.40	0.50	0.2050	0.0026
Aa15	AF469499	F^: TCACTGACCTGCCCTCTACA R: CCAACCCTCTAGTCGTCCAC	$(CA)_{13}$	197–203	3	0.66**	0.53	0.0081	0.0009
Aa26	AF469501	F^: GCAAAGGTAAACTGCATCTGG R: ATGCACTATTGGTAAACAGGCA	$(AC)_{14}$	145–159	5	0.58	0.64	0.4952	0.0124
Aa27	AF469502	F^: GAGATGTCTTCACAGCTTGGC R: AAGTCTCAGAGACTGACGGACC	$(CA)_{11}$	92–98	3	0.53	0.58	0.3451	0.0052
Aa35	AF469503	F^: GCAGCAGAAAGTGCATACGA R: GACCAAATGAAATGCGCC	$(AC)_{17}$	250–264	5	0.45	0.40	0.1408	0.0101
Aa36	AF469504	F^: ACAGGCCAGCACCAAGAG R: TTTGGAGCCATTGTTACCGT	$(AC)_{16}$	109–119	5	0.47*	0.62	0.0420	0.0040
Aa39	AF469505	F^: TTCTGTTTTTCCACTTGCTTG R: TATTGAGCTCACAAAAACAAAGG	$(AC)_{13}$	191–223	7	0.53**	0.77	0.0057	0.0013
Aa41	AF469507	F^: CCAGCAGGCACCTGTTTTAT R: AAAAGTTTGGGCATTTGTGG	$(CAAA)_9$	151–159	4	0.42*	0.61	0.0487	0.0041
Aa43	AF469508	F^: CCACACTGAGAAACTCCTGTTG R: TTCCTGAGAGCTCTTCCTGC	$(AC)_{14}$	108–114	4	0.18**	0.30	0.0063	0.0015
Aa49	AF469509	F^: AGGAGGTGCCAGTTTTCTCC R: AGCGGGTCTGTGGCTCAT	$(AC)_{12}$	146–156	5	0.45*	0.65	0.0183	0.0024
Aa50	AF469510	F^: AACATGGCAATGTGTTTCGA R: ATTGACGCTGCAAACAGATG	$(TG)_{11}$	209–219	4	0.63	0.57	0.5874	0.0074
Aa51	AF469511	F^: CCAGGAAAATGACTGTGGCT R: GTTCCTGGATGTTCACTTCCA	$(TG)_{11}$	230–232	2	0.42	0.47	0.7279	0.0018
Aa53	AF469512	F^: ATCGCTTCCATGAGCTGATT R: GAGTGCGGAGAGCTCTGC	$(CA)_{12}$	123–133	5	0.34***	0.63	0.0001	0.0001
Aa56	AF469513	F^: GGGGTGAAACACAGATGCTT R: CAAGCAACTGGCAACTTGAA	$(GT)_{14}$	249–263	6	0.42*	0.46	0.0493	0.0071
Aa57	AF469514	F^: AACATTAAGGCAGATGTGGACA R: TACTGTGGACACGGACAGGA	$(TG)_{12}$	113–115	2	0.29	0.32	0.6086	0.0018

Exact test of Hardy–Weinberg equilibrium showed significant heterozygote deficits in some loci (*P < 0.05; **P < 0.01; ***P < 0.001).
^ indicates fluorescently labelled primer.

step at 94 °C for 2 min; 17 cycles of 92 °C for 30 s, annealing at 66–50 °C for 30 s (1 °C decrease in each cycle), and extension at 72 °C for 30 s; 19 cycles of 92 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. A final extension was programmed at 72 °C for 5 mn. Fragments were analysed in an ABI 310 Genetic Analyser (Applied Biosystems). Allele scoring was done with Genotyper 2.5 software (Applied Biosystems). Observed and expected heterozygosities and paternity exclusionary power were estimated with the program cervus (Marshall *et al*. 1998). Linkage disequilibrium and Hardy–Weinberg equilibrium were tested with the probability test of genepop (http://wbiomed.curtin.edu.au/genepop/index.html (Raymond & Rousset 1995). Probability of identity (PID) and probability of identity of full siblings (PID_{sib}) were estimated as described in Waits *et al.* 2001).

The mean number of alleles per locus among Spanish imperial eagles was 4.0 (Table 1). Expected heterozygosities ranged from 0.29 to 0.76. Only 10 of the loci conformed to Hardy–Weinberg expectations when tested with the probability test of genepop (Table 1). Evidence for linkage disequilibrium between some pairs of loci was found in Spanish imperial eagle, but not in the eastern imperial eagle, *A. heliaca* (the sister species). The total exclusionary power in parentage analysis of these loci is 0.951 when both parents are unknown and 0.998 when one of them is known

Table 2 Cross amplification of the 18 microsatellite markers in five raptor species. Some of the loci are fixed for some species. Number of individuals typed is shown in parenthesis when different from n. *** means no amplification product was obtained. None of the products have been sequenced

	Aquila heliaca (*n* = 18)				*Aquila chrysaetos* (*n* = 6)				*Aquila nipalensis* (*n* = 5)				*Hieraaetus pennatus* (*n* = 8)				*Haliaeetus vociferoides* (*n* = 8)			
Locus	Size range	No. of alleles	H_O	H_E	Size range	No. of alleles	H_O	H_E	Size range	No. of alleles	H_O	H_E	Size range	No. of alleles	H_O	H_E	Size range	No. of alleles	H_O	H_E
Aa02	139–157	9	0.72	0.80	135–141	4	0.67	0.71	***	***			133–147	4	0.75	0.68	131	1	—	—
Aa04	124–132	4	0.78	0.65	122–150	6	0.50	0.68	124–136	4	0.20	0.8	125–131	2 (5)	0.20	0.20	114	1 (6)	—	—
Aa11	244–252	3	0.17	0.16	257–263	2	0.33	0.30	252	1 (4)	—	—	253–261	3 (7)	0.71	0.54	243–245	2 (7)	0.57	0.44
Aa12	133–141	3	0.44	0.52	***	***			138–150	3	0.80	0.53	124	1	—	—	131–133	2	—	0.23
Aa15	197–203	4	0.56	0.72	199–201	2	0.50	0.41	196–206	4 (3)	1.00	0.87	198–204	4	0.63	0.70	193	1 (7)	—	—
Aa26	147–149	2	0.61	0.51	137–151	4	0.50	0.65	148–156	4	0.80	0.78	148	1	—	—	133–135	2	—	0.23
Aa27	86–98	4	0.33	0.53	84–96	3 (5)	0.40	0.38	88–94	2	0.40	0.36	82	1	—	—	86–88	2	—	0.23
Aa35	248–276	11	0.89	0.88	230–256	4	0.50	0.74	269–275	4	0.80	0.80	***	***			239	1	—	—
Aa36	109–123	5	0.67	0.76	92–102	3	0.67	0.53	107–127	6	0.80	0.89	***	***			112–114	2	0.25	0.23
Aa39	184–202	9	0.78	0.87	185–201	6	0.67	0.88	168–192	5	0.40	0.53	179–203	7 (7)	1.00	0.85	***	***		
Aa41	147–155	3	0.39	0.53	151–153	2	0.33	0.30	138–150	3	0.80	0.60	***	***			***	***		
Aa43	106–116	5	0.61	0.70	106–130	5	0.83	0.79	106–114	5	0.80	0.76	105–111	3	0.75	0.68	90–92	2	—	0.23
Aa49	146–156	5	0.61	0.72	137–149	4	0.17	0.80	130–150	5	0.80	0.84	152–166	5	0.38	0.68	142–146	3	0.13	0.34
Aa50	215–217	2	0.11	0.11	209–215	2	0.33	0.30	215	1	—	—	208	1	—	—	218	1	—	—
Aa51	230–232	2	0.33	0.41	232–234	2	0.17	0.1	228–232	3	0.60	0.51	226–236	5	0.63	0.68	230	1 (7)	—	—
Aa53	125–133	5	0.78	0.77	120–122	2	0.17	0.17	127–137	6	1.00	0.89	125–135	4	0.75	0.66	118	1	—	—
Aa56	249–267	8	0.89	0.80	251	1	—	—	237–255	4	0.60	0.73	243	1	—	—	236	1	—	—
Aa57	107–117	5	0.72	0.79	***	***			111–115	3	0.60	0.60	113	1	—	—	123–125	2	0.50	0.50

(Marshall *et al*. 1998). PID and PID_{sib} are 2.9×10^{-11} and 1.8×10^{-5}, respectively.

A cross amplification test was performed in five other raptor species spanning from the sister taxon, the eastern imperial eagle (*A. heliaca*), two other species of the same genus, the golden eagle (*A. chrysaetos*) and the steppe eagle (*A. nipalensis*) and two species from different genera, the booted eagle (*Hieraaetus pennatus*) and the madagascar fish eagle (*Haliaeetus vociferoides*) (Table 2).

The microsatellite markers reported here provide powerful tools for population genetic studies, paternity assessment and the unambigous identification of individuals for ecological and forensic applications for the Spanish imperial eagle and related species.

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