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PRIMER NOTE

Isolation and characterization of microsatellite markers in Bonelli's eagle (*Hieraaetus fasciatus*)

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Abstract

To obtain polymorphic molecular markers for population genetics and conservation studies in Bonelli's eagle populations, we screened a partial genomic library enriched for microsatellites with di- and tri-nucleotide motifs [(GT) (CT) (AAC) and (GCC)]. A total of 15 polymorphic markers were obtained. The number of alleles ranged from two to eight. These markers will be very useful for paternity tests and population structure studies as well as for evaluating the outcome of conservation programs.

Keywords: Bonelli's eagle, enriched libraries, microsatellite markers

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In Europe, the Bonelli's eagle is restricted to the Mediterranean area and considered an endangered species. Currently, its population size is estimated at about 860–1000 couples. In the last decades, overall populations from the Mediterranean borders have shown a decline of up to 50% of their original numbers, leading to local extinction due to persecution and habitat destruction.

Endangered species suffering a significant reduction in population size, such as the Bonelli's eagle, will suffer a corresponding decrease in genetic variability which may reduce the evolutionary potential of the populations. Investigating the genetic diversity of this species could help to evaluate the current status of the different populations. For this, microsatellites have proven to be a powerful tool.

Genomic DNA was extracted from blood samples following the protocol described in Seutin *et al*. (1991), and approximately 10 μg was used to construct a partial enriched genomic library.

Total genomic DNA was digested with *Afa*I (Amersham Biosciences) and the resulting fragments were linked to annealed *Afa*I adapters (AdapF: 5′-TCTTGCTTACGCGTGGACTA-3′ and AdapR: 5′-TAGTCCACGCGTAAGCAAGAG-3′) according to Edwards *et al*. (1996). We followed the protocol of Billotte *et al*. (1999) with some modifications. Three microlitres of DNA with linkers were used for the preamplification step using AdaptF as a primer in a 20-cycle polymerase chain reaction (PCR). After purification through columns (Amersham Biosciences), the resulting PCR product was used to start the enrichment process using streptavidin-coated magnetic spheres (Promega), as suggested by Kijas *et al*. (1994). Four different probes, $(GT)_{15}$, $(CT)_{15}$ $(AAC)_{10}$ and $(GCC)_{10}$, were used in independent reactions, all of them modified at both extremities (5′-biotinylated and 3′-dideoxyC) to avoid the production of chimeric clones (Koblízková *et al*. 1998). A double enrichment was performed on each case to increase the efficiency of the library. The enriched ssDNA was then amplified by PCR using the AdapF to recover double-stranded DNA, and the resulting purified products were directly cloned into pGEM-T easy vector (Promega) and transformed into DH5α competent cells.

A total of 1615 positive clones (570 GT, 475 CT, 285 AAC, and 285 GCC) were incubated 4 h at 37 °C on 96 well microtitre plates with 150 μL of LB/ampicilin solution. We screened the library by dot-blotting the lysed bacteria (4× dilution) onto Hybond N + membranes (Hybond-XL — Amersham Biosciences) and hybridizing with γ^{32}P radio-labelled probes [$(GT)_{15}$ $(CT)_{15}$ $(AAC)_{10}$ and $(GCC)_{10}$].

About 27% of the clones gave a positive signal [28% (GT), 20% (CT), 20% (AAC), and 49% (GCC)], and from these, 115 were sequenced using ABI PRISM BigDye Terminator (version 1.1) cycle sequencing ready reaction kits (Applied Biosystems) and either detected on an automated sequencer (ABI 310 PRISM®), or sent to Macrogen (Korea). Based on the identified sequences, 40 pairs of primers were designed using PRIMER version 3.0 software (Rozen & Skaletsky 2000).

The primer pairs were tested for polymorphism in 10 juveniles, nestlings sampled on the northeast of Portugal.

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Table 1 *Hieraaetus fasciatus* microsatellite loci. Locus, Accession Nos., primers (F — Forward and R — Reverse), repeat motif, and size range of the amplified products are indicated. Type of test (radioactivity labelled — rad-lab, fluorescent labelling — ABI) and multiplex indicated by M1 and M2 (multiplex 1 or multiplex 2), $MgCl_2$ concentrations, annealing temperature (T_m), number of alleles obtained along with the observed (H_O) and nonbiased expected (H_{NB}) heterozygosities are also indicated. Numbers of alleles and heterozygosities are based on 10 juvenile samples

Locus	Accession Nos.	Primers (5′–3′)	Repeat motif	Size (bp)	Test type/ Multiplex	$MgCl_2$ (mM)	T_m	Nb alleles	H_O–H_{NB}	*P* value
Hf-C1E8	AY823587	F: ATTGGCAGGTCTTGGA R: CTCTAATGAAACCAGAAAGA	$(GAA)_{26}$	262–280	rad-lab	2	54	3	0.4–0.57	0.20
Hf-C1E6	AY823586	F: ACATGGGAGGGTAGTGGG R: AATGAAGAAATACTGTTTGC	$(GAA)_{15}GAG(GAA)_{16}$	234–243	rad-lab	1.8	60	4	0.5–0.54	0.53
Hf-C1D10	AY823588	F: TGGCAGCTCAGTTCTTTA R: CGTTGGAGGAATAATGAG	$(GAA)_{19}$	189–195	rad-lab	1.8	55	3	0.3–0.28	0.83
Hf-C7E1	AY823592	F: GTCTCTTGACTCTCCCTTC R: GGAGCCAATAAGTTCTGC	$(GA)_{22}$	158–170	rad-lab	2	50	5	0.8–0.77	0.60
Hf-P2E11	AY823585	F: AATAGGATGAAGGCTGAA R: ATTTCTCTCTCTCCTCTCTT	$(TG)_{15}$	93–95	rad-lab	2	52	2	0.1–0.1	0.29
Hf-C4G1	AY823589	F: AATCCCTGACCAAAATGT R: GTCCTTCTGTTTGCCTGA	$(AG)_{17}$	220–227	rad-lab	2	53	3	0.4–0.42	0.81
Hf-C11D4	AY823593	F: TCACTAGAAACGGGGCACTT R: TGGCGCAATCATCTTCAATA	$(TC)_{12}$	245–247	ABI 310/ M1	2	58/55	2	0.33–0.5	0.39
Hf-C2D4	AY823595	F: CAGCAAGAAAGAAAAAGTTCAG R: GCTTCACTGCAATCTTTGGTG	$(GA)_{15}$	163–189	ABI 310/ M1	2	58/55	4	0.7–0.71	0.56
Hf-C5D4	AY823597	F: GGACAAACCCAGGTCATGTT R: TTTCTGGAGTCAGGCAATTACA	$(GA)_{18}$	174–186	ABI 310/ M1	2	58/55	4	0.7–0.64	0.51
Hf-C7G4	AY823598	F: TGTGAGCAGTTTTTAGGGAACA R: AAGCAGATGTTGGCAGGAAT	$(GA)_{11}TA(GA)_7$	149–161	ABI 310/ M1	2	58/55	3	0.6–0.67	0.41
Hf-C8F4	AY823599	F: GCAACCTCAGCTCTCTTTTGA R: CATCTTTTGGTGGCTTCCAT	$(GA)_{14}$	244–251	ABI 310/ M1	2	58/55	3	0.2–0.19	0.93
Hf-P1A10	AY823584	F: CTTCACTGCAGAGGTTAAAA R: GGCAATTTAAGATATAGTAC	$(GT)_{14}(GA)_{22}$	249–289	ABI 310/ M2	2	54/50	6	0.5–0.58	0.33
Hf-C6C4	AY823591	F: GTGACACCCACAAAAC R: ATGCTGCTCTGAATAAGT	$(GA)_{28}$	159–201	ABI 310/ M2	2	54/50	8	0.9–0.86	0.58
Hf-C1D2	AY823594	F: TGTAACAATTGAAAATGAAATTGGA R: CCTTCCTGACACCTGGAAAA	$(AG)_{20}$	161–181	ABI 310/ M2	2	54/50	4	0.6–0.61	0.58
Hf-C3F2	AY823596	F: TGCTTGGCTTGTTAATTCCA R: TCTCCTTCCTTAGCTGAAGACAC	$(CT)_{20}$	175–196	ABI 310/ M2	2	54/50	6	0.8–0.83	0.51

The PCRs were conducted in a total volume of 10 μL containing 50 ng of DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, between 1.8 and 2.0 mM $MgCl_2$, 10 μM dATP, 60 μM of each of the other dNTPs, 0.5 μM of each primer, 0.5 units of *Taq* DNA polymerase (Invitrogen), and 0.08 μL of [α-^{35}S]dATP (12.5 mCi/mL, 1250 Ci/nmol). Reactions were performed in a Stratagene Robocycler and consisted of a first denaturation step at 94 °C for 4 min, followed by 30 cycles of denaturing for 45 s at 94 °C, annealing for 45 s at 50–60 °C (see Table 1), and extension for 45 s at 72 °C. A final extension of 5 min at 72 °C was performed. The PCR products were size separated over a denaturing 6% acrylamide-bisacrylamide gel and visualized by autoradiography. Some primers were tested for polymorphism by fluorescence analysis (see Table 1) using an automated sequencer (ABI 310 Prism®) and PCRs were performed in a final reaction volume of 10 μL, containing 1× buffer supplied, 2.0 mM $MgCl_2$, 0.2 mM each dNTP, 0.20 μM each primer, 0.6 U *Taq* (Bioline) under the following conditions: 94 °C for 8 min, 20 cycles at 94 °C for 15 s, 53 °C for 15 s, 72 °C for 15 s followed by 10 cycles at 89 °C for 15 s, 50 °C for 15 s, 72 °C for 15 s and then 72 °C for 12 min, in a MJ Research PTC-100 (MJ Research).

Fifteen loci revealed polymorphism (Table 1) and those analysed on the ABI310 were optimized for multiplexing (monomorphic primer pairs are available in Genbank under Accession Nos. AY823590 and AY823600–AY823618). Observed and expected heterozygosities were calculated using GENETIX (Belkhir *et al.*), and no departure from Hardy–Weinberg equilibrium was found (Table 1), as well as no linkage disequilibrium between any pair of loci. We also tested some primers described by Martinez-Cruz and collaborators (2002), and Aa02, Aa50, Aa39, and Aa26 are polymorphic for this species.

The polymorphism found with these 15 primers is sufficient to undertake studies in population structure and for paternity tests in the endangered Bonelli's eagle.

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Reference

Belkhir K, Borsa P, Goudet J, Chikhi L, Bohnomme F *(GENETIX) Genetix Logiciel sous Windows™ pour la Génétique des Populations*. URL http://www.univ-montp2.fr/~genetix/genetix/genetix.htm Accessed: 6th April 2005.

Billotte N, Lagoda PJL, Risterucci AM, Baurens FC (1999) Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. *Fruits*, **54**, 277–288.

Edwards KJ, Barker JHA, Dal A, Jones C, Karp A (1996) Microsatellite libraries enriched for several microsatellite sequences in plants. *BioTechniques*, **20**, 758–760.

Kijas JMH, Fowler JCS, Garbett CA, Thomas MR (1994) Enrichment of microsatellites from the citrus genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. *BioTechniques*, **16**, 657–662.

Koblízková A, Dolezel J, Macas J (1998) Subtraction with 3' modified oligonucleotides eliminates amplification artifacts in DNA libraries enriched for microsatellites. *BioTechniques*, **25**, 32–38.

Martinez-Cruz B, David VA, Godoy JA, Negro JJ, O'Brien SJ, Johnson WE (2002) Eighteen polymorphic microsatellite markers for the highly endangered Spanish imperial eagle (*Aquila adalberti*) and related species. *Molecular Ecology*, **2**, 323–326.

Seutin G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology*, **69**, 82–90.

Rozen S, Skaletsky HJ (2000) PRIMER3 on the www for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, NJ. Source code available at http://fokker.wi.mit.edu/primer3/. Accessed: 6th April 2005.