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Isolation and characterization of polymorphic microsatellite markers in Eurasian vulture *Gyps fulvus*

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Abstract

To obtain polymorphic molecular markers for population genetic and conservation studies in the Eurasian vulture *Gyps fulvus* populations, we screened a size-selected partial genomic library enriched for microsatellites with dinucleotide motifs. A total of five polymorphic markers were obtained. The number of alleles ranged from two to nine and the observed and expected heterozygosity were very similar. These markers will be very useful for studying population structure and to evaluate conservation programmes.

Keywords: Eurasian Vulture, *Gyps fulvus*, microsatellite

During the last century the Eurasian vulture *Gyps fulvus* was widely distributed in the western palearctic, particularly around the Mediterranean area (Sarrazin *et al.* 2000). However a dramatic decline occurred around the end of the 19th century and beginning of the 20th century, leading to the conservation of Eurasian Vulture populations in many countries particularly in Europe (Sarrazin *et al.* 2000).

Population genetics play an important role in the development of conservation programmes for endangered species. Recently the use of microsatellites as molecular markers has proved to be an excellent tool for genetic studies (Jarne & Lagoda 1996). Here we present the development of microsatellite markers that can be useful for developing and monitoring conservation and reintroduction programmes of *G. fulvus*.

Genomic DNA was extracted from blood samples following the protocol described in Seutin *et al.* (1991) and used to construct a partial enriched genomic library following protocols slightly modified from Edwards *et al.* (1996) and Kijas *et al.* (1994). DNA was digested with the restriction enzyme *Sau3AI* and the resulting fragments, ranging from 200 to 800 bp, were ligated into annealed *Sau3AI* adaptors (AdapF: 5'-CTCTTGCTTACGCGTGGACTC-3'

and AdapR: 5'-GATCGAGTCCACGCGTAAGCAAGAG-CACA-3'). After denaturation, single stranded DNA was enriched through hybridization with 5'-biotinylated, 3'-aminated (CT)₁₅ oligonucleotide bound to streptavidin-coated magnetic spheres (Magnisphere, Promega, Madison, WI, USA), following Koblízková *et al.* (1998). The enriched DNA was then used as template for amplification using the polymerase chain reaction (PCR) with AdapF as the primer to recover double stranded DNA fragments. The resulting purified PCR product (8 ng) was directly ligated overnight at 4 °C into pGEM-T easy vector (50 ng) (Promega, Madison, WI, USA) using 3 units of T4 ligase (Promega, Madison, WI). One tenth of the ligation product was used to transform 100 µL of supercompetent cells (XII-Blue MRF', Stratagene, La Jolla, CA). Libraries were screened using PCR performed directly on the lysed bacteria, using a (CT)₁₀ primer and a plasmid primer (forward or reverse), according to Waldbieser (1995).

Out of 800 bacterial clones, 172 gave a positive result. 45 positive clones were sequenced according to the dideoxy chain termination method (Sanger *et al.* 1977) and 22 were found to contain a microsatellite. For 10 of the clones containing at least four consecutive repeated motives and where the position of the microsatellite would allow, primers were designed for the flanking regions using the PRIMER 3.0 software (Rozen & Skaletsky 1997). Polymorphism was tested on 10 juvenile vultures migrating through Portugal and held for rehabilitation in the Natural Park of Ria Formosa (two individuals in 1994 and eight in 1999). PCR analysis was performed in a total volume of

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Table 1 *Gyps fulvus* microsatellite loci. The locus, accession number, number of motif repeat, primers, and size of the amplification product from the clones are indicated, annealing temperature, MgCl₂ concentrations, 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 a sample of 10 juveniles migrating through Portugal

Locus	Accession number	Motif	Primer (5' → 3') F: Forward; R: Reverse	Size (bp)	Anneal. Temp (°C)	MgCl ₂ (mM)	No of alleles	H_O	H_{nb}
Gf11A4	AY035858	(CTCTT) ₁₇	F: GATCCCTTCCAACCGAAAAT R: TGGTGACCAACGGAAGTGTG	148	50	2	7	1.00	0.85
Gf3H3	AY035859	(CT) ₁₂	F: GTAGAATAATTGCTCCTGG R: GTGAAGGCACCTCATAGACA	139	48	2.5	5	0.70	0.76
Gf3F3	AY035860	(CT) ₁₀	F: GATCTTTCCCTTCTGTG R: TTCGTGCAGTGATGCTGGTG	180	52	2.5	2	0.30	0.39
Gf8G1	AY113692	(CT) ₈ C(TC) ₂	F: TGAGCAGGTGAGTCCAGAAG R: GCTCTCCTGTCATCTTGCAT	271	50	2	2	0.50	0.39
Gf9C1	AY035855	(TC) ₁₀₊ (CT) ₉ C (CA) ₅ T(AC) ₄	F: GGTGGACATTACATACACTG R: CAAGGAATCTGGACTACTAA	255	50	2	9	0.80	0.87

10 µL containing 10–50 ng of DNA, 20 mM Tris-HCl (PH 8,4), 50 mM KCl, between 2.0 and 2.5 mM MgCl₂, 10 µM dATP, 60 µM of each of the other dNTPs, 0.5 µM of each primer, 0.5 unit of *Taq* DNA polymerase (Gibco BRL, Life Technologie Inc., Gaithersburg, MD) and 0.08 µL [α -³⁵S]dATP (12.5 mCi/mL, 1250 Ci/nmol). Reactions were conducted in a Stratagene Robocycler (Stratagene Cloning Systems, La Jolla, CA) 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 48 °C up to 52 °C (depending on the locus, see Table 1), and extension for 45 s at 72 °C. A final extension period of 5 min at 72 °C was performed. PCR products were size fractionated over a denaturing 6% acrylamide-bisacrylamide gel and visualized using autoradiography.

Of the 10 microsatellites isolated five corresponded to polymorphic loci exhibiting from two to seven alleles (Table 1). These molecular markers were also tested on genomic DNA from Bonelli's eagle (*Hieraetus fasciatus*) but only locus Gf3H3 was found to be polymorphic with 2 alleles; the rest being monomorphic.

Observed and expected heterozygosities calculated using GENETIX (Belkhir *et al.* 2000), were similar (Table 1), and no linkage disequilibrium was detected between each pair of locus.

In conclusion, we developed five microsatellite markers for the Eurasian Vulture *G. fulvus* that are appropriate molecular tools for population genetic studies and for designing strategies in conservation and reintroduction projects.

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