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Brief Communication

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Isolation of Microsatellite Loci by Next-Generation Sequencing of the Critically Endangered Northern Bald Ibis, *Geronticus eremita*

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Abstract

The Northern Bald Ibis is one of the rarest bird species, extinct in Europe for 400 years and critically endangered worldwide. The European Union-co-financed LIFE+ project "Reason for Hope - Reintroduction of the Northern Bald Ibis in Europe" aims to reintroduce the species in Europe (Germany, Austria, Italy). In order to obtain information on the genetic diversity within zoo colonies and the reintroduced population, 15 polymorphic microsatellite markers, specific for the Northern Bald Ibis, *Geronticus eremita* (Linnaeus, 1785), have been isolated from next-generation sequencing (Illumina MiSeq) and are described here. The microsatellite primers were tested in 30 individuals and measures of genetic variability were calculated. Values for the observed heterozygosity ranged from 0.393 to 0.867, while expected heterozygosity ranged from 0.573 to 0.718. Ten out of 15 loci were in Hardy–Weinberg equilibrium and only one showed indication for the presence of null alleles. The newly developed PCR primers can be used to examine population genetic parameters, e.g. for future conservation genetic studies of this critically endangered bird species.

Subject areas: Conservation genetics and biodiversity
Keywords: conservation genetics, captive breeding, *Geronticus eremita*, microsatellite, reintroduction.

The Northern Bald Ibis, *Geronticus eremita* (Linnaeus 1758) is a migratory bird belonging to the family of Threskiornithidae. Its former distribution area ranged from the European Alps to North Africa and the Middle East (Bowden et al. 2008). In Europe, the Northern Bald Ibis is extinct in the wild. The remaining wild or semi-wild populations outside Europe occur in the western (Morocco) and eastern Mediterranean (Syria and Turkey) (BirdLife International 2015). Since 2012, the Northern Bald Ibis is classified as Critically Endangered on the IUCN Red List of Threatened Species (BirdLife International 2013). Currently, about 1300 individuals are kept in European zoos. The last known wild population in Morocco comprises about 524 individuals (Grepom BirdLife

Table 1. Characterization of 15 polymorphic microsatellite primers for *Geronticus eremita* with locus name; repeat motif; primer sequence of forward (F) and reverse (R) primer; fluorescence dye name (Tag); multiplex reaction (MPR); allele size range; number of successfully genotyped individuals (*N*); annealing temperature (T_a); number of alleles per locus (N_a); observed heterozygosity (H_o); expected heterozygosity (H_e); significance of departure from Hardy–Weinberg equilibrium (HWE)

Locus	Repeat motif	Primer sequence (5′–3′)	Tag	MPR	Allele size range (bp)	*N*	T_a	N_a	H_0	H_e	HWE	NullF
A02	$(GATAGA)_{12}$	F:GCTTGAAGTGAAGGTCCTATGG R:TCAGTCTAGGTGAAGGTGCC	FAM	5	292–322	30	59 °C	6	0.667	0.767		0.064
A06	$(TAGAA)_{13}$	F:CTGCAACTGGAAACTGGTAGG R:GTTCAAAGCTGCACCAGGG	HEX	3	338–358	30	61 °C	5	0.690	0.670		−0.019
A07	$(ATAGA)_{12}$	F:CTGACACCAGGAAAGATGGC R:GGATCCAGTGCCAGGATATG	FAM	1	163–188	30	59 °C	6	0.433	0.483	***	0.067
A09	$(TAAGGG)_{10}$	F:ACTTCCCTGGTGAACTGC R:GAGGGCTTACAGTGACCAAC	HEX	1	228–252	30	59 °C	5	0.800	0.779		−0.017
A11	$(TTTGC)_{11}$	F:CCTGAGGACAGCAGGGTC R:TTCCGTAAGACGGGACAGC	FAM	2	300–320	30	61 °C	5	0.867	0.718		−0.111
A12	$(GATG)_{14}$	F:GGACATTCAACAAGGCCGAG R:GGCTCATGTCCCGTTTGTC	HEX	3	177–193	30	61 °C	5	0.533	0.677	***	0.109
B01	$(ATAGA)_{11}$	F:TTGCCTGTTGAGGTTTGCC R:CTTGGACCCTAGCCTCTGC	FAM	4	310–325	30	59 °C	4	0.586	0.710		0.084
B04	$(CATC)_{12}$	F:TTCGCAAACGTCGAGCTTC R:TGACAATCAACTCAGGGCTC	HEX	4	342–358	30	59 °C	5	0.533	0.663	***	0.092
B05	$(CTATT)_9$	F:TTTGGTCAGCCCTGAAGCG R:ACTCAGGATCGCATTTCACC	FAM	2	189–204	30	61 °C	4	0.667	0.668		−0.013
B06	$(AATAG)_9$	F:TGCCATGTCCCTACCTTGG R:TCCGGCAGTTGGACTAGATG	FAM	5	209–234	30	59 °C	5	0.533	0.686		0.116
B08	$(AGAT)_{12}$	F:GTGTCCCTTTCAGGATGGTG R:AGGCTTTCTCACACTGGGC	FAM	3	232–252	30	61 °C	5	0.393	0.573		0.156*
B11	$(GTGA)_{11}$	F:CCCTGGGAACAACGTGAATG R:GGTGGGAAGGCAGCAAATC	HEX	5	255–263	30	59 °C	3	0.500	0.620	*	0.087
B12	$(ATTCT)_8$	F:GGACTCGGCAGTGCTAGG R:GCTGCCTGCTGTGTTTACC	HEX	2	265–285	30	61 °C	5	0.633	0.667	**	0.038
C02	$(GTTTG)_8$	F:TCTAAACAGCTTCCAAGGTCTG R:CCAGTTGATGTACCGTCGC	HEX	4	228–243	30	59 °C	4	0.467	0.636		0.134
D07	$(CAAA)_8$	F:TCCTTGTTTGTTAGACTACTGCC R:GCAGCTGGGTTGGGTTATC	FAM	1	324–336	30	59 °C	4	0.433	0.526		0.086

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); estimated null allele frequency (NullF) according to van Oosterhout et al. (2004).

Maroc 2014). The Northern Bald Ibis lives year-round in colonies (Böhm and Pegoraro 2011). Its breeding sites are niches in sheer rock walls, often close to coastal areas or rivers (Cramp et al. 1977), castle ruins, and niches in former town walls (in Böhm and Pegoraro 2011 after Gesner 1557; Unsöld and Fritz 2011). The colonies are often affiliated to agricultural land. The EU-LIFE + project "Reason for Hope – Reintroduction of Northern Bald Ibis in Europe" currently reintroduces the species in its former habitats in the Alps (Austria, Switzerland, and Germany; Fritz and Unsöld 2015). Genetic analyses are particularly important in the context of reintroduction, as it is necessary to analyse the genetic variability of the founders, avoid inbreeding and ensure a good likelihood of persistence. Polymorphic microsatellites have become prevalent as a marker of choice in such population genetic studies (Selkoe and Toonen 2006; Witzenberger and Hochkirch 2011), but up to now no specific microsatellite-based PCR assays for the Northern Bald Ibis are known.

Material and Methods

Blood and salivary samples from 10 captive-bred, 5 semicaptive, and 1 wild individuals were obtained to create a genomic DNA library (Supplementary Table S1). DNA was isolated from blood samples using the DNeasy Blood & Tissue Kit according to the manufacturer's protocol. About 1 µg genomic DNA was used to construct a DNA sequencing library according to the Illumina TruSeq v.2 kit protocol (www. illumina.com). Essentially, the DNA was sheared to an average size of 700 bp, repaired by A-tailing, ligated to TruSEq adaptors and amplified by 10 PCR cycles. An average integrate length of 580 bp was achieved. Sequencing was done in the 2 × 300 bp paired-end mode on an Illumina MiSeq system, and 56.2 million paired-end reads (in total yielding 12.53 gb) were generated. Overlapping paired-end reads were assembled using the tool FLASH (Magoc and Salzberg 2011). The generated sequences were screened for microsatellite loci. A total of 64 loci were selected using the criteria microsatellite type (≥ trinucleotide) and length (≥ 6 repeat units), rate of heterozygosity, as well as the position of the microsatellite within the sequence (Supplementary Table S2). A total of 48 loci produced interpretable results when conducting the polymerase chain reaction (PCR). The concentrations for a 25-µL PCR reaction were: 1 µL template DNA (20 ng), 1 µL primer forward and reverse (10 pmol/µL), 2 µL dNTPs (2.5 mM), 5 µL 5× PCR buffer, 2 µL $MgCl_2$ (25 mM), 0.1 µL Taq-Polymerase (1.25 Units), and 12.9 µL Aqua bidest. PCR conditions were as follows: initial denaturation 3 min at 95 °C, 34 cycles (denaturation: 30 s at 95 °C, annealing: 30 s at 55 °C, elongation: 30 s at 72 °C) and final elongation of 5 min at 72 °C.

Data Availability

In accordance with the Journal of Heredity data archiving policy (Baker 2013), primary data underlying these analyses (origin, sex, and age of individuals and microsatellite genotypes) are deposited at Dryad Digital Repository.

Results and Discussion

Out of the 48 loci, we selected 15 loci that showed highest polymorphism. These primers were labelled using FAM and HEX fluorescent dye and pooled in 5 multiplex reactions, which were tested in 30 individuals (Supplementary Table S3). Multiplex PCR reactions of 55 µL were mixed as follows: 1 µL template DNA, 5 µL forward and reverse primers (2 pmol/µL), 25 µL Qiagen Multiplex PCR Master Mix, 5 µL Q-Solution, and 14 µL RNase-free water. PCR was done in a Labnet thermocycler under the following conditions: initial denaturation 15 min at 95 °C, 34 cycles (denaturation: 30 s at 94 °C, annealing: 90 s at 59 °C/61 °C depending upon the locus (see Table 1), elongation: 60 s at 72 °C) and final elongation of 30 min at 60 °C. Fragment length analysis was conducted on an ABI 3500 sequencer. The data were scored with the software Genemapper 5.0 (genotype data can be found in the Supplementary Table S3 as well as in the Dryad Digital Repository). For the detection of potential scoring errors (e.g. null alleles, stutter bands and large allele dropout), the data was analysed with Micro-Checker 2.2.1 (van Oosterhout et al. 2004). FSTAT 2.9.3.2 (Goudet 2001) was used to test for linkage disequilibrium. Population genetic parameters were calculated in GenAlEx 6.5 (Peakall and Smouse 2006).

The number of alleles per locus varied from 3 to 6 (Table 1). Values for observed and expected heterozygosity ranged from 0.393 to 0.867 and 0.573 to 0.718, respectively. The loci A7, A12, B04, B11, and B12 deviated significantly from Hardy–Weinberg equilibrium (Table 1). This may be caused either by our sampling as the tested individuals stemmed from different source populations (i.e. a Wahlund Effect) or by non-detected null alleles (Waples 2015). None of the loci showed evidence for large allele dropout or stutter bands, but locus B08 showed evidence for null alleles. The test for linkage disequilibrium between all pairs of loci, showed no significant results. The set of microsatellite markers developed in this study can serve as a useful tool to gain information about the genetic status of the zoo populations of the Northern Bald Ibis and provides guidance for the reintroduction process. Furthermore, it is also useful for future studies on free ranging populations or parentship analyses.

Supplementary Material

Supplementary material can be found at https://jhered.oxfordjournals.org/.

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Data Availability

Data deposited at Dryad: http://dx.doi.org/doi:10.5061/dryad.h1167

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