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Research Article

Genomic resources for the conservation and management of the harpy eagle (*Harpia harpyja*, Falconiformes, Accipitridae)

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Abstract

We report the characterization and optimization of 45 heterologous microsatellite loci, and the development of a new set of molecular sex markers for the conservation and management of the Neotropical harpy eagle (*Harpia harpyja* L. 1758). Of the 45 microsatellites tested, 24 were polymorphic, six monomorphic, 10 uncharacterizable due to multiple bands and five did not amplify. The observed gene diversity of the analyzed sample of *H. harpyja* was low and similar to that of other threatened Falconiformes. While a high proportion of the microsatellite markers were highly variable, individuals of *H. harpyja* could be differentiated by a joint analysis of just three ($p = 2.79 \times 10^{-4}$) or four markers ($p = 2.89 \times 10^{-5}$). Paternity could be rejected with 95.23% and 97.83% probabilities using the same three and four markers, respectively. The sex determination markers easily and consistently differentiated males from females even with highly degraded DNA extracted from naturally shed feathers. The markers reported in this study potentially provide an excellent set of molecular tools for the conservation and management of wild and captive *H. harpyja* and they may also prove useful for the enigmatic Neotropical crested eagle (*Morphnus guianensis* Daudin 1800).

Key words: conservation genetics, *Harpia harpyja*, microsatellites, raptors, sex markers.

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Introduction

The Neotropical harpy eagle (*Harpia harpyja* L. 1758, Falconiformes, Accipitridae) is the largest eagle in the Americas and is considered the most powerful bird of prey in the world (Collar, 1989; Sick, 1997). This species inhabits the upper stratum of New World forests from southern Mexico to northeastern Argentina but is comparatively rare throughout its distribution. The main threats to the conservation of *H. harpyja* is habitat fragmentation, hunting and trade in live birds (Vargas G *et al.*, 2006). The slow reproductive rate and low population densities of *H. harpyja* make these threats significant throughout its distribution. *Harpia harpyja* is classified as near threatened by the International Union for the Conservation of Nature (IUCN) and is cited in Appendix I of the Convention on the International Trade of Threatened Species of Fauna and Flora (CITES). While active conservation programs exist in several countries where this eagle occurs, implementation of conservation programs is challenging and conservation success is difficult to assess due to the difficulty of obtaining ecological data. Molecular markers often allow indirect estimates of many ecologically important parameters, and if available would greatly facilitate the conservation and management of *H. harpyja*.

The knowledge of biology of threatened species is of indispensable interest for conservation. However, as in the case for many threatened raptors, *H. harpyja* is difficult to study because adults are very difficult to capture and mark, individual birds may move over great distances and while slight size dimorphism exists, there is no sexual dimorphism in plumage making males and females difficult-to-distinguish. However, molecular tools may overcome many of these challenges (*e.g.* Frankham *et al.*, 2002; Allendorf and Luikart, 2006), especially since such methods can use non-invasive sampling techniques based on DNA extraction from feathers (*e.g.* Pearce *et al.*, 1997; Segelbacher, 2002; Horváth *et al.*, 2005; Rudnick *et al.*, 2005). Understanding the genetic characteristics of a species is also extremely important for the success of *in situ*

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and *ex situ* conservation programs because this information allows definition of management units needed to minimize the loss of genetic diversity while at the same time preserving the existing genetic structure of the species (Haig, 1998; Hedrick, 2001).

Of the various types of molecular markers used today, microsatellites have many positive attributes, including hypervariability, co-dominance, abundance and tolerance to variation in DNA quality and quantity (Selkoe and Toonen, 2006). Additionally, due to our reasonably good understanding of molecular evolution and the development of robust computational methods, microsatellites are well-suited to answer questions related to effective population size, population structure, migration and colonization rates, and reproductive system, thus providing essential data for conservation. The need to characterize species-specific loci by expensive and laborious isolation and characterization procedures is the primary limitation to the more widespread use of microsatellites. Although mutations in the flanking regions of microsatellite loci may prevent amplification, many studies have shown that microsatellites isolated from one species can amplify homologous products in related species (*e.g.* Martínez-Cruz *et al.*, 2002; Busch *et al.*, 2005), a characteristic known as transferability or cross-species amplification (Selkoe and Toonen, 2006).

Other important molecular markers for conservation are those for sex determination, mainly for species that do not possess apparent sexual dimorphism, whether at the juvenile or adult stage, as is the case of for *H. harpyja*. In all neognath birds, the female is the heterogametic (WZ) and the male homogametic (ZZ) sex. Molecular methods of sex determination are based on the amplification of paralogous copies of the Chromo helicase DNA binding protein gene (*CHD1*) present on chromosomes W and Z using mismatch primers, *i.e.* while the forward primer amplifies *CHD1* copies on both W and Z chromosomes, the reverse primers are designed to anneal to either W or Z chromosome and amplify different sized products. Molecular sex markers developed for birds (Griffiths *et al.*, 1998) have limited applicability in Falconiformes due to the fact that the differentiation of amplified *CHD1*W and *CHD1*Z fragments is unreliable because both fragments are large and similar in size. Recent publication by Ito *et al.* (2003) presents a solution that appears to be applicable to all Falconiformes by increasing the size difference of the *CHD1*W and *CHD1*Z amplified fragments. However, the relatively large size of these fragments makes PCR amplification from forensic samples difficult.

In this study we present the results of an amplification test of 45 microsatellite loci, isolated and characterized by other authors in various other accipitrid raptors, the characterization and optimization of 30 of these microsatellites and the development of a new set of molecular sex markers for *H. harpyja*. In addition, we also present a preliminary characterization of genetic diversity of *H. harpyja* and comment on the usefulness of a subset of these microsatellite markers for assessing the joint probability of the identity of any two samples and of paternity exclusion. We conclude that a carefully chosen subset of microsatellite markers optimized for multiplexing and the newly developed molecular sex markers provide highly valuable and simple-to-use set of molecular tools to assist in the formulation of conservation and management strategies for this threatened raptor.

Material and Methods

Specimens

Molted feathers were collected from individual harpy eagle (*Harpia harpyja* L. 1758 Falconiformes, Accipitridae) specimens from three main Brazilian biomes: the Amazonian rainforest, the Atlantic rainforest and the Pantanal wetland. Some feather samples were collected from nests in the wild while other samples came from zoos and museums; however, in all cases samples of feather originated from wild-born individuals (Table 1).

Microsatellite loci

There are approximately 100 microsatellite loci isolated and characterized for Falconiformes, some of these being published after we commenced our study. For testing in *H. harpyja* we chose 45 based in the following criteria: 1) loci were isolated from related falconiform species, 2) loci had at least five alleles in the species for which they were developed, and had preferentially a perfect repeat motif, and 3) when cross-species amplification tests were made, the loci were polymorphic in phylogenetically distantly related taxa. The tested microsatellite loci were taken from six microsatellite panels described by the following authors: Nesje and Røed (2000); Martínez-Cruz *et al.* (2002); Busch *et al.* (2005); Hailer *et al.* (2005); Johnson *et al.* (2005); Mira *et al.* (2005); a complete list of loci is presented in Table 2. Preliminary screening was done using two captive *H. harpyja* specimens for which sufficient quantities of good quality DNA could be extracted. For this preliminary screening and for characterization of specimens in Table 1, total genomic DNA was extracted from a blood clot in the superior umbilicus (a small opening at the proximal tip of the calamus or quill) as recommended by Horváth *et al.*, (2005) using the Qiagen® DNA extraction kit (Valencia, CA, USA). Polymerase chain reactions (PCR) were carried out a total volume of 10 µL consisting of 1 µL of sample DNA (~10 ng), 1 µL each of forward and reverse primer (2 µM), 1 µL of 10X Buffer (200 mM Tris-KCl, pH 8.5), 0.7 µL of $MgCl_2$ (25 mM), 0.8 µL of dNTP (10 mM), 0.2 µL *Taq* DNA polymerase (5 units/µL; Biotools, Spain) and 4.3 µL of water. All primers were purchased from IDT, Coralville, IA, USA (www.idtdna.com), and dNTPs were purchased from Fermentas, Glen Burnie,

Table 1 - Demographic information for the harpy eagles (*Harpia harpyja*) subjected to microsatellite primer characterization. All birds originated in the wild.

Original source location (biome, municipality and state)	Specimen code	Specimen origin	Institution providing the sample[†]
Amazon			
Parintins, Amazonas	H2	Nature	INPA Gavião-real Project
Manaus, Amazonas	H3	Nature	INPA Gavião-real Project
Amazonas	H14	Nature	INPA Gavião-real Project
Tailândia, Pará	H5	Nature	INPA Gavião-real Project
Belterra, Pará	H6	Nature	INPA Gavião-real Project
Labrea, Amazonas	H4	Captive	CIGS Zoo
Amazonas	Hh1	Captive	CIGS Zoo
Amazonas	Hh2	Captive	CIGS Zoo
Amazonas	Hh3	Captive	CIGS Zoo
Amazonas	Hh4	Captive	CIGS Zoo
Amazonas	Hh5	Captive	CIGS Zoo
Costa Marques, Rodônia	H7	Museum	IBAMA Museum, Costa Marques, Rondônia
Atlantic forest			
Eunápolis, Bahia	H11	Captive	Breeder, Águia Branca, Espírito Santo state
Bahia	H12	Captive	Estação Vera Cruz/Veracel, Porto Seguro, Bahia
Foz do Iguaçu, Paraná	H27	Captive	Bela Vista Biological refuge, Foz do Iguaçu, Paraná
Cascavél, Paraná	H28	Museum [-]	Natural History Museum, Capão do Imbuia, Curitiba, Paraná
Pantanal			
Bonito, Mato Grosso do Sul	H30	Nature	Gerencia do PARNA da Serra Bodoquena

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[-]Accession number MHNCI 2918.

MD, USA (www.fermentas.com). Amplification consisted of 35 cycles of denaturation at 93 °C for 15 s, annealing between 50 °C and 55 °C for 30 s and extension at 68 °C for 30 s, and a final seven minute extension at 68 °C was added after the last cycle. For the microsatellite loci that amplified we re-synthesized the forward primer by adding an M13 tail to its 5' end to allow for dynamic fluorescent labeling with FAM-6 labeled M13 primer following the protocol described by Schuelke (2000). Genotyping PCR was performed in a total volume of 10 µL containing 1 µL of reverse primer (0.2 µM), 0.5 µL of M13 labeled forward primer (0.2 µM), 0.5 µL of FAM-6 labeled M13 primer (0.2 µM) and the other reagents described above. Amplification was carried out in a Hybaid PCR thermocycler (Thermo Scientific, USA) and had two main cycling steps (modified from Schuelke, 2000), consisting of an initial denaturation step of 1 min at 93 °C followed by 30 cycles of 30 s at 93 °C, 30 s at 55 °C and 30 s at 68 °C then 20 cycles of 30 s at 93 °C, 30 s at 50 °C, and 30 s at 68 °C. The reaction was completed by a final extension for 30 min at 68 °C to minimize stutter due to non-specific incorporation of adenine (Brownstein *et al.*, 1996). The PCR product was visualized using a MegaBACE1000 (GE Healthcare, United Kingdom) and analyzed with the software Fragment Profiler v1.2 (GE Healthcare, United Kingdom) following the manufacturer's recommendations. For each microsatellite marker we genotyped 10 to 17 (average 15) specimens of *H. harpyja* originating from all three main Brazilian biomes (Table 1). The variable number of specimens analyzed per microsatellite locus was due to failures in genotyping and the limited quantity of DNA available for repeat analyses, a common problem with forensic samples such as naturally shed feathers (Segelbacher, 2002).

The characterization of each microsatellite locus was based on number of alleles and gene diversity (Nei, 1978), expected (H_E) and observed (H_O) heterozygosity (Weir, 1996), deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium between all pairs of loci. All the analyses were performed using the program Arlequin v3.1 (Excoffier *et al.*, 2005), with significance levels for multiple tests being adjusted using the sequential Bonferroni correction (Rice, 1989). To evaluate the potential use of the microsatellite loci for relatedness analyses, we also estimated the probability of paternity exclusion at an individual locus (Q or P_{ei}), and the joint probability of paternity exclusion at all loci (QC or P_{et}) following Weir (1996). Additionally, we estimated the probability of genetic identity at an individual locus (I) and the joint probability of genetic identity at all loci (IC) according to Paetkau *et al.* (1995).

Table 2 - Characterization of 45 microsatellite loci for the harpy eagle (*Harpia harpyja*) originally isolated from other raptor species by the authors cited. Loci which failed to amplify are underlined and a dash (-) indicates loci that failed to genotype. Table shows the following: number of eagles tested (N); number of alleles per locus, with the range of allele sizes in base pairs in parentheses (A); observed heterozygosity (H_O); expected heterozygosity (H_E); significance of the difference between H_O and H_E (only the microsatellite BBU46 showed significant deviation after Bonferroni correction for multiple comparisons) (p); probability of paternity exclusion (Q); and probability of genetic identity (I).

Author and locus	GenBank numbers	Repeat motif	*H. harpyja*						
			N	*A*	H_O	H_E	*p*	*Q*	*I*
Nesje and Røed, 2000									
NVHfr142	AF200201	$(GT)_{12}$	-	-	-	-	-	-	-
NVHfr144-2	AF200202	$(CA)_{15}$	-	-	-	-	-	-	-
NVHfr190-2	AF200204	$(CA)_{12}$	-	-	-	-	-	-	-
NVHfr195-2	AF200205	$(CA)_{16}$	15	2 (154-160)	0.07143	0.07143	1.00000	0.03383	0.86941
NVHfr206	AF200207	$(CA)_{14}$	17	7 (156-166)	0.66666	0.76782	0.57515	0.54547	0.10344
Martínez-Cruz *et al.*, 2002									
Aa11	AF469497	$(CA)_{11}$	13	3 (244-248)	0.38462	0.33354	1.00000	0.16622	0.49190
Aa12	AF469498	$(GT)_{12}$	-	-	-	-	-	-	-
Aa26	AF469501	$(AC)_{14}$	15	2 (133-135)	0.20000	0.18621	1.00000	0.08595	0.68860
Aa27	AF469502	$(CA)_{11}$	17	1 (87)	-	-	-	-	-
Aa36	AF469504	$(AC)_{16}$	16	4 (93-119)	0.53333	0.66322	0.12511	0.37763	0.21334
Aa43	AF469508	$(AC)_{14}$	16	7 (101-115)	0.93333	0.76552	0.21817	0.53864	0.10823
Aa49	AF469509	$(AC)_{12}$	11	1 (146)	-	-	-	-	-
Aa57	AF469514	$(TG)_{12}$	16	5 (120-130)	0.80000	0.66437	0.42135	0.42738	0.17271
Busch *et al.*, 2005									
IEAAAG04	AY631063	$(AAAG)_6(AAAC)_4(AAAG)_6$	13	7 (216-244)	0.61538	0.81538	0.00655	0.60251	0.07861
IEAAAG05	AY631064	$(AAAG)_7$	11	1 (108)	-	-	-	-	-
IEAAAG11	AY631066	$(AAAG)_{26}$	-	-	-	-	-	-	-
IEAAAG12	AY631067	$(AAAG)_{10}(GAAG)_3(AAAG)_5$	-	-	-	-	-	-	-
IEAAAG14	AY631069	$(AAAG)_{18}$	-	-	-	-	-	-	-
IEAAAG15	AY631070	$(AAAG)_7$	17	11 (136-176)	1.00000	0.89425	0.01247	0.74009	0.03279
Hailer *et al.*, 2005									
HAL01	AY817040	$(GT)_{17}$	15	2 (116-118)	0.06667	0.18621	0.10360	0.08595	0.68860
HAL03	AY817042	$(CAAA)_6$	15	2 (141-145)	0.85714	0.50794	0.02233	0.21491	0.38025
HAL04	AY817043	$(CA)_2AA(CA)_{12}CG(CA)_4$	16	2 (156-158)	0.06667	0.06667	1.00000	0.03170	0.87735
HAL09	AY817048	$(AC)_{17}$	16	4 (131-137)	0.73333	0.57241	0.28994	0.29392	0.29251
HAL10	AY817049	$(CA)_{12}$	17	3 (217-221)	0.33333	0.29655	1.00000	0.14708	0.53511
HAL13	AY817052	$(CA)_{17}$	-	-	-	-	-	-	-
Johnson *et al*., 2005									
BBU42	AJ715912	$(GGGT)_5(GA)_5$	15	2 (204-206)	0.07143	0.07143	1.00000	0.03383	0.86941
BBU46	AJ715916	$(AC)_{12}$	16	4 (147-153)	1.00000	0.54943	0.00030	0.25831	0.32979
BBU06	AJ715878	$(AC)_9$	10	1 (97)	-	-	-	-	-
BBU33	AJ715903	$(GT)_{12}$	-	-	-	-	-	-	-
BBU34	AJ715904	$(AC)_{12}$	-	-	-	-	-	-	-
BBU51	AJ715921	$(AC)_{17}$	14	3 (150-154)	0.35714	0.31481	1.00000	0.15609	0.51429
BBU59	AJ715928	$(CA)_5$	16	1 (132)	-	-	-	-	-
Mira *et al.*, 2005									
HF-C1D2	AY823594	$(AG)_{20}$	16	7 (167-179)	0.93333	0.71724	0.00632	0.48810	0.13549
HF-C1D10	AY823588	$(GAA)_{19}$	-	-	-	-	-	-	-
HF-C1E6	AY823586	$(GAA)_{15}GAG(GAA)_{16}$	10	3 (167-185)	0.30000	0.48947	0.00217	0.26298	0.33134
HF-C1E8	AY823587	$(GAA)_{26}$	17	5 (216-231)	0.73333	0.68046	0.16898	0.42061	0.18058
HF-C2D4	AY823595	$(GA)_{15}$	-	-	-	-	-	-	-
HF-C3F2	AY823596	$(CT)_{20}$	13	4 (165-171)	0.38462	0.67385	0.01486	0.41819	0.17951
HF-C4G1	AY823589	$(AG)_{17}$	-	-	-	-	-	-	-
HF-C5D4	AY823597	$(GA)_{18}$	16	5 (168-176)	0.53333	0.48276	0.65557	0.27000	0.32718
HF-C6C4	AY823591	$(GA)_{28}$	15	1 (134)	-	-	-	-	-
HF-C7E1	AY823592	$(GA)_{22}$	14	2 (144-146)	0.23077	0.21231	1.00000	0.09686	0.65424
HF-C7G4	AY823598	$(GA)_{11}TA(GA)_7$	16	3 (115-139)	0.33333	0.38391	0.60033	0.18736	0.44392
HF-C8F4	AY823599	$(GA)_{14}$	-	-	-	-	-	-	-
HF-P1A10	AY823584	$(GT)_{14}(GA)_{22}$	-	-	-	-	-	-	-

Sex determination markers in *H. harpyja*

For the characterization of sex markers we used the primers developed by Ito *et al.* (2003). Tests were performed on 10 specimens of *H. harpyja* collected from museums, zoos and nature (Table 3), of which two samples were of know sex while the other samples were of unknown sex. The PCR reactions for the sex markers were carried out in a total volume of 25 μL containing 1 μL of DNA (~10 ng), 2.5 μL of NP primer (2 μM) 1.3 μL of MP primer (2 μM), 1.3 μL of P2 primer (2 μM), 2.5 μL of 10X Buffer (200 mM Tris-KCl, pH 8.5), 2.5 μL of $MgCl_2$ (25 mM), 2.0 μL of dNTP (10 mM), 0.2 μL *Taq* DNA polymerase (5 units/μL; Biotools, Spain) and 11.8 μL of deionized water. All primers were purchased from IDT, Coralville, IA, USA (www.idtdna.com), and dNTPs were purchased from Fermentas, Glen Burnie, MD, USA (www.fermentas.com). The thermocycling profile consisted of 1 min denaturation at 93 °C, followed by 35 cycles of denaturation at 93 °C for 10 s, annealing at 52 °C for 35 s and extension at 68 °C for 30 s. The reaction was completed by a final extension for seven minutes at 68 °C. The PCR products were separated on a 3% (w/v) agarose gel. To assess the consistency of the results we repeated each PCR three times for each specimen. The *CDH1Z* and *CDH1W* gene fragments of a number of the specimens did not amplify, most likely due to a high degree of DNA degradation; therefore we designed two primers, CHD1Wr (5'-GCTGATCTGGTTTCAGAT TAA-3') and CHD1Zr (5'-AGTCACTATCAGATCCAG AG-3') as substitutes for primers MP (Ito *et al.*, 2003) and P2 (Griffiths *et al.*, 1998) respectively (Table 4). Our new primer set reduced the size of amplicons by nearly 100 bp and using this strategy we were able to sex the remaining specimens of unknown sex.

Results and Discussion

Transferability and characterization of the microsatellites in *H. harpyja*

Of the 45 microsatellite loci tested in our sample of *H. harpyja*, 40 amplified successfully but only 30 could be genotyped unambiguously. All 30 loci amplified at 55 °C and produced unambiguous genotypes, thus all PCR reactions were standardized to this annealing temperature. A total of 24 microsatellites loci were polymorphic and the number of alleles per locus ranged from 2 to 11 (Table 4). After sequential Bonferroni correction for multiple comparisons (Rice, 1989), a significant departure from HWE was observed only in the locus BBU46. This and six additional loci that showed HWE deviations before Bonforroni

Table 3 - Harpy eagle individuals (*Harpia harpyja*) subjected in the sex-determination.

Specimen code	Specimen origin	Institution providing the sample[†]	Sex
1 = H8	Captive	UFMT Zoo	female
2 = H121	Captive	Bosque Municipal de São José do Rio Preto, São Paulo	female
3 = H56*	Museum[-]	INPA Coleção de Aves	male
4 = H57*	Museum[¯]	INPA Coleção de Aves	female
5 = H71	Captive	Foz Tropicana Parque das Aves, Foz do Iguaçu, Paraná	female
6 = H75	Captive	Bioparque Amazônia Crocodilo Safari Zoo, Belém, Pará	female
7 = H76	Captive	Bioparque Amazônia Crocodilo Safari Zoo, Belém, Pará	male
8 = H79	Captive	Parque Zoobotânico do Museu Paraense Emilio Goeldi, Belém, Pará	female
9 = H96	Captive	Criadouro Conservacionista Sítio Tibagi, Serra Guaramiranga, Ceará	female
10 = H120	Captive	UFMT Zoo	male

[†]INPA = Instituto Nacional de Pesquisas da Amazônia, Manaus, AM; UFMT = Universidade Federal do Mato Grosso, Cuiabá, MT.
*Control individuals of known sex. [-]Accession number INPA 629. [¯]Accession number INPA 829.

Table 4 - Primers used for molecular sex determination of the harpy eagle (*Harpia harpyja*).

Primer	5' - 3' primer sequence	Author
P2 (anneals to CHD1W/Z)	TCTGCATCGCTAAATCCTTT	(Griffiths *et al.*, 1998)
MP (anneals to CHD1W)	AGTCACTATCAGATCCAGAA	(Ito *et al.*, 2003)[*]
NP (anneals to CHD1W/Z)	GAGAAACTGTGCAAAACAG	(Ito *et al.*, 2003)
CHD1Wr (anneals to CHD1W)	GCTGATCTGGTTTCAGATTAA	This study
CHD1Zr (anneals to CHD1Z)	AGTCACTATCAGATCCAGAG	This study

*Ito *et al.* (2003) report the sequence of the primer MP as 5'-AGTCACTATCAGATCCGGAA-3'; however this clearly is a mistake as can be seen from Figure 2 of their paper and GenBank sequences AB096141-AB096156.

correction were re-scored from original electrophoregrams to eliminate potential scoring biases. The observed and expected gene diversity (Nei, 1978) over all loci was 0.50580 and 0.47242, respectively. We found less than 5% of pairs with significant linkage disequilibrium across all pairs of loci. Indexes of joint probability of paternity exclusion ($p = 0.99596$) and genetic identity ($p = 1.04221 \times 10^{-8}$) were highly robust.

The high rate of cross-species amplification (40 out of 45 loci) and characterizability (30 out of 45 loci) was attributable to our initial choice of loci. Five of the six microsatellite panels were developed for other Accipitrid species, the same family as the *H. harpyja*. The transferability of microsatellite primers between species is directly related to the genetic divergence of the species concerned; the greater the genetic divergence, the greater the probability of mutations at priming sites, and thus lower the probability of successful annealing of primers. Although we chose only polymorphic loci, the rates of polymorphism characterized for *H. harpyja* did not reflect the polymorphism observed in the original studies (reg. $R = 0.035$, $p = 0.402$). Levels of polymorphism depend on the sample analyzed, and there is also no expectation of transferability of the degree of polymorphism (Ellegren *et al.*, 1995).

The 24 polymorphic loci appeared to present an excellent panel for populational analyses of *H. harpyja*. They were also robust markers for estimating kinship and paternity relations (Weir, 1996) and to identify individuals (Paetkau and Strobeck, 1995). However, statistically significant levels of paternity exclusion and genetic identity can be obtained with a panel of only three or four loci. Paternity can be excluded at the $p = 0.95233$ and genetic identity rejected at the $p = 0.00028$ levels using just the loci IEAAAG15, IEAAAG04 and Aa43. The addition of the NVHfr206 locus would increase these probabilities to $p = 0.97834$ and $p = 0.00003$ levels, respectively. The allelic classes produced by the loci IEAAAG15, IEAAAG04 and Aa43 are non-overlapping, and thus conducive to multiplexing even with one dye set and dynamic fluorescent labeling of alleles (Schuelke, 2000). Inclusion of the locus NVHfr206 would require the use of a second fluorescent dye since its allele sizes overlap with those of the locus IEAAAG15. Dynamic multiplexing with the first three loci would result in a statistically significant estimate of paternity exclusion and/or genetic identity at less than US$ 1 per sample analyzed.

Genetic diversity of *H. harpyja*

For the IUCN near-threatened *H. harpyja* the average H_O calculated by us was 0.506, similar to that for the recently surveyed accipitrid species *Aquila adalberti* (the Spanish imperial eagle; $H_O = 0.516$) and *Aquila heliaca* (the eastern imperial eagle; $H_O = 0.563$) listed as vulnerable by IUCN (Martinez-Cruz *et al.*, 2004). Genetic diversity is necessary for populations and species to adapt to environmental change and reflects their evolutionary potential (Frankham *et al.*, 2002), low genetic diversity therefore being viewed as an indirect measure of extinction threat. It may also be that *H. harpyja* presents the signature of a genetic bottleneck. Garza and Williamson (2001) have demonstrated that for a population sample of microsatellite loci the mean ratio of the number of alleles to the range in allele size, the *M* parameter, can be used to detect reductions in population size. The average value of *M* for the 24 microsatellite loci was 0.84, a value significantly lower than that obtained under simulation of a pre-bottleneck population size ($p = 0.026$ using the genetic parameter θ of 2.24). θ is a summary statistic representing four times the product of the effective population size and the mutation rate (Hartl and Clark, 1997). We derived θ from estimated census sizes of 10^4 to 10^5 harpy eagle individuals (Ferguson-Lee, 2001; Vargas G *et al.*, 2006) assuming that the effective number of individuals is equivalent to 1/10 the census size (Frankham *et al.*, 2002), and that microsatellite mutation rate (μ) estimates range from 2.5×10^{-3} to 5.6×10^{-4} (*e.g.* Dallas, 1992; Weber and Wong, 1993; Brinkmann *et al.*, 1998; Sajantila *et al.*, 1999; Kayser and Sajantila, 2001; Hrbek *et al.*, 2006). Using the most conservative parameter estimates ($\theta = 2.24$, $\mu = 5.6 \times 10^{-4}$) a value of $M = 0.84$ reflects a significant reduction in population size ($p = 0.026$). When the parameter θ was estimated directly from the microsatellite data ($\theta = 1.50$), the *M* value was not significant ($p = 0.101$). However, the θ calculated from the data itself is necessarily a lower bound estimate if *H. harpyja* shows any population structure. Although there is a possibility that *H. harpyja* has experienced a genetic bottleneck, a more definitive inference can only be made with more extensive sampling, and the determination of any existing population structuring.

Although we cannot extend the perceived threat to the entire distribution of *H. harpyja*, it seems reasonable to extrapolate low genetic diversity and associated threat potential to other Neotropical regions which are often anthropogenically impacted and near the periphery of the natural distribution of this raptor. Within Brazil, *H. harpyja* possesses its core and broadest area of distribution and, at least within the Amazon basin, it appears to have suffered limited anthropogenic impact. Yet, the genetic diversity of *H. harpyja* is lower than that of other accipitrid raptors listed by IUCN in categories which indicate a greater risk of extinction. Furthermore, *H. harpyja* is not even on the official list of threatened species of the Brazilian Environmental Agency (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis - IBAMA). Although our preliminary data clearly bring into question the current classification status by IUCN and IBAMA, in order to facilitate adequate management and conservation policies, a denser sampling throughout the distribution of *H. harpyja* is necessary to find out how genetic diversity is distributed

over geographic landscape, how genetically diverse is *H. harpyja* throughout the areas of its distribution, and if it has suffered a genetic bottleneck.

Molecular sex markers

Using the primers from Ito *et al.* (2003) we were able to confirm the sex of the two *H. harpyja* specimens of known sex, a male (specimen 3) and a female (specimen 4) (Figure 1). The PCR pattern of males is characterized by a single band and that of females by two bands, with 100 base pairs difference between the two bands. The other eight *H. harpyja* specimens of differing DNA qualities and concentrations were characterized as two males (specimens 7 and 10) and six females (specimens 1, 2, 5, 6, 8 and 9). Molecular sex determination was repeated three times, each time resulting in the same pattern. The primer set of Ito *et al.* (2003) minimizes false positive identifications since the female-specific *CHD1W* gene product is the smaller product. Theoretically even in the case when highly degraded DNA is used and only the smaller *CHD1W* gene fragment is amplified, this fragment will 100 bp smaller than the *CHD1Z* gene fragment and therefore this specimen will be easily identifiable as a female. However, some of our specimens showed no amplification of the molecular sex markers, most probably due to the very high levels of DNA degradation common in feathers, which apparently do not allow amplification of the ~300 to 400 bp fragments generated by the markers from Ito *et al.* (2003). Therefore we designed primers CHD1Wr and CHD1Zr to substitutes for primers MP (Ito *et al.*, 2003) and P2 (Griffiths *et al.*, 1998), respectively in our PCR reactions. The new primer combination NP/CHD1Wr produced a 250 bp fragment while NP/CHD1Zr produced a 300 bp fragment. Primers to obtain even shorter products could not be designed, since the regions amplified span a size variable intron lacking suitably conserved regions. With these new primer combinations we were able to sex our remaining specimens.

Molecular tools for the conservation of the harpy eagle

We have characterized a set of molecular tools useful for *in situ* and *ex situ* conservation and management of *H. harpyja*. The loci IEAAAG15, IEAAAG04 and Aa43 together with sex markers provide powerful and cost effective tools for identifying best potential mates in captive breeding programs. The correct identification of the sex of individual birds in conservation programs, currently a difficult invasive procedure, is clearly fundamental for the success of any breeding program. If the goal of the breeding program is to minimize pedigree inbreeding and maximize genetic diversity, microsatellite markers in addition to the three presented above will need to be used. Captive breeding decisions must be made in light of any potential natural population genetic structuring which, although at present unknown, will be easily determinable with the presented set

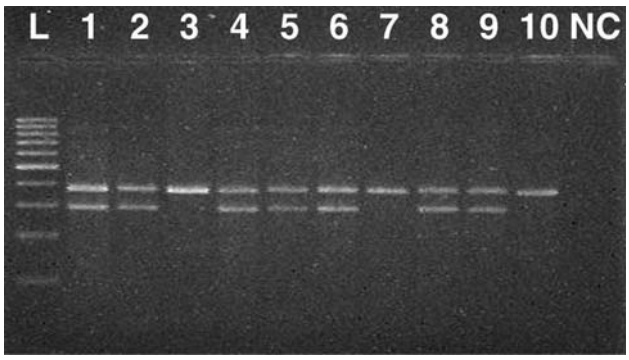

Figure 1 - Molecular discrimination of *Harpia harpyja* specimens of known and unknown sex. Individuals 3 and 4 are male and female, respectively. Females show two bands separated by approximately 100 bp while males show only a single band. The size standard (L) is the GeneRuler 100 bp DNA ladder (Fermentas, Hanover, MD, USA). Negative control is labeled as NC.

of 24 polymorphic microsatellite markers once sufficient sampling data are obtained. The existence, or absence, of population structure is also critical for *ex situ* management and reintroduction programs. With few exceptions, *H. harpyja* is effectively extinct in the Brazilian Atlantic rainforest and IBAMA has approved a plan presented by the CRAX Society (Sociedade de Pesquisa do Manejo e Reprodução da Fauna Silvestre, MG, Brazil) to reintroduce *H. harpyja* into the Atlantic Rainforest from a captive population maintained and bred by the CRAX Society. The captive population consists of birds from various biomes, confiscated animals of unknown origin and the hybrid offspring of these animals (Nemésio *et al.*, 2000). The program is currently stalled as specific areas of introduction and financial sponsors have yet to be identified. Even more critically, no data currently exist on whether *H. harpyja* from the Amazon rainforest and the Atlantic rainforest form one large population and are genetically and demographically interchangeable, or if they represent two differentiated populations. The introduction of inappropriate birds could have serious negative conservation consequences, potentially even leading to the extinction of the remnant Atlantic rainforest populations of *H. harpyja* (Frankham *et al.*, 2002; Hedrick, 2005; Allendorf and Luikart, 2006). If, however, introductions are scientifically justified, they would be of great benefit in helping to rescue the highly threatened Atlantic rainforest population. Therefore a molecular study of representative specimens from the Amazon and Atlantic rainforests is urgently needed, and the markers reported in this study will greatly facilitate these conservation efforts. Last, but not least, if *H. harpyja* shows a signal of strong population structuring, these microsatellites could further be used to identify the origin of seized specimens from illegal animal traders and clandestine breeding units. In Brazil, for instance, confiscated specimens of *H. harpyja* are generally destined for zoos and legalized breeding units (Efe *et al.*, 2006), the principal reason for this bureaucratic decision being the lack of knowl-

edge of the region in which the individuals were clandestinely captured. The assignment of confiscated specimens to their regions of origin could be accomplished with the use of molecular methods, and the confiscated birds could be repatriated to their areas of origin. However, in the case that *H. harpyja* comprises a panmitic population there will be no restrictions on seized specimens being released in any region within the distribution of *H. harpyja* and these specimens may be used to augment the severely depleted Atlantic rainforest population. The caveat of these inferences is that they are based on neutral genetic markers. It is possible that *H. harpyja* from different areas of its distribution may show adaptive differences even if differentiation among regions is not observed at the level of neutral genetic markers, and therefore management and conservation strategies should not solely rely on conclusions drawn from putatively neutral microsatellite markers. In spite of this cautionary note, we believe that the markers reported in this study will prove to be excellent tools for the conservation and management of *H. harpyja* throughout its geographic distribution, and we also presume that they can be utilized for studies of other raptor species such as the enigmatic species of the genus *Morphnus*.

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