Sex identification of neotropical macaws (*Ara* spp.) from invasive and non-invasive samples

Identificación del sexo en guacamayas neotropicales (*Ara* spp.) a partir de muestras invasivas y no invasivas

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Abstract

While amplification of the Chromo-Helicase-DNA-binding gene (CHD) from blood and feathers has been used to sex macaws, the performance of non-invasive samples has been poorly explored. We optimized a comprehensive protocol of molecular sexing of macaws *(Ara* spp.) from blood, plucked feathers and fecal samples, using the 2550F/2718R primers to amplify the CHD gene. This protocol is versatile and useful for studies in both ex situ and in situ settings. We successfully identified the sex of macaws from the three types of tissue. However, we recommend sexing from fresh fecal pellets to minimize physical restriction, stress and risk of injury on animals.

Key words: CHD gene, non-invasive sampling, Psittacidae, sex chromosomes, sexing.

Resumen

Aunque la amplificación del gen Helicasa con Cromodominio de Unión a ADN (CHD) ha sido usado para inferir el sexo en guacamayas a partir de sangre y plumas, el uso de muestras no invasivas ha sido poco explorado. Aquí optimizamos un protocolo completo de identificación molecular del sexo en guacamayas *(Ara* spp.) a partir de muestras de sangre, plumas arrancadas y muestras fecales, utilizando los primers 2550F/2718R para amplificar el gen CHD. Este protocolo es versátil y de utilidad para estudios tanto ex situ como in situ. Logramos identificar exitosamente el sexo de las guacamayas a partir de los tres tipos de tejidos muestreados. Sin embargo, recomendamos el uso de deposiciones fecales frescas para minimizar la manipulación, el estrés y el riesgo de lesión de los animales.

Palabras clave: CHD, cromosomas sexuales, muestreo no invasivo, identificación del sexo, Psittacidae.

The limited sexual dimorphism of most psittacine birds (parrots and macaws) is an obstacle in the identification of sexes and establishment of breeding programs in captivity. A way to tackle this problem is the differential PCR amplification of sex chromosomes in males (ZZ) and females (ZW). Specifically, the Chromo-Helicase-DNA-binding gene (CHD) has non-recombining copies of unequal size in the Z (CHD-Z) and W (CHD-W) chromosomes, whose amplification yields different profiles in males and females (Dvorák *et al.* 1992, Ellegren 1996, Griffiths & Tiwari 1995, Griffiths *et al.* 1996, Matta *et al.* 2009).

Even though fecal material provides an easy, safe and direct access to genetic material, its advantage over invasive samples such as blood and feathers in sexing psittacine birds has been poorly assessed (Griffiths & Tiwari 1995, Robertson *et al.* 1999, Jensen *et al.* 2003, Ong & Vellayan 2008). Isolation of amplificable DNA from non-invasive samples such as feces is nonetheless challenging due to its low quality and concentration, high rate of genotyping errors, and limited amplification success (Taberlet *et al.* 1996, Gagneux *et al.* 1997, Robertson & Gemmell 2006, Bosnjak *et al.* 2013).

Table 1. Sample sizes, tissues and inferred sex in six species of *Ara.*

Species	n	Ft, B and Fc	Ft and B	Only Ft	Only B	Males / Females
A. ambiguus	24	6 *	12	6	-	15/9
A. militaris	3	-	-	-	3	3/0
A. ararauna	1	-	-	1	-	1/0
A. ararauna x A. chloropterus	1	-	-	1	-	0/1
A. chloropterus	1	-	-	1	-	1/0
A. macao	3	-	-	3	-	0/3
Total	33	6	12	12	3	20/13

DNA was obtained from plucked feathers (Ft), blood (B) and feces (Fc).

* These include two controls (one male and one female).

The aim of the present study was to optimize and provide a flexible and reliable approach to genotype the CHD gene in macaws (genus *Ara)* from invasive (blood), moderately invasive (plucked feathers) and non-invasive samples (feces). This strategy fits a wide diversity of locations and technical circumstances for sexing macaws both in situ and ex situ.

To do this, we took blood, plucked feathers and fecal samples from 33 specimens of five of the nine recognized species of Ara (IUCN 2017) and a hybrid. We collected samples of 24 Great Green Macaw *(Ara ambiguus),* including a breeding couple of known sex that were used as sexing controls (Table 1 and Supplementary Table). We also obtained blood or plucked feathers from three Military Macaws *(A. militaris)*, one Blue-and-Yellow Macaw *(A. ararauna),* one Red-and-Green Macaw *(A. chloropterus),* three Scarlet Macaws *(A. macao)* and one hybrid "harlequin macaw" *(A. ararauna* x *A. chloropterus).* Blood samples were collected from the brachial vein and stored in EDTA at 4°C. Two feathers were plucked from the chest and stored in dry ziplock plastic bags at 4° C. Fresh feces (<24 h) were gathered from the ground in the zoo habitat where birds are held or collected directly from the cloaca using a sterile cotton bud. Samples were provided by the Santa Fe Zoological Park or kindly donated by JL Parra and HF Rivera.

We extracted DNA from 10 ml of blood using the DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany). We chopped the calamus of two feathers (2 mm approximately) into fine pieces and then extracted the DNA using the same kit. Also, we extracted the DNA from fecal material (180 mg approximately) using the Fast DNA Stool Mini kit (QIAamp, Hilden, Germany). In all cases we followed the protocols recommended by the manufacturer. We performed independent amplifications of the CHD gene with the primer pairs 2550F (5'GTTACTGATTCGTCTACGAGA3') and 2718R (5'ATTGAAATGATCCAGTGCTTG3') described by Fridolfsson & Ellegren (1999), and alternatively with P2 (5'TCTGCATCGCTAAATCCTTT3') and P8 (5'CTCCCAAGGATGAGRAAYTG3') (Griffiths *et al.* 1998). The primers 2550F/2718R are expected to allow the amplification of fragments between 400 and 450 bp in the CHD-W, and between 600 and 650 bp in the CHD-Z (Fridolfsson & Ellegren 1999), whereas the P2/P8 target shorter regions in the CHD-W (380-410 bp) and the CHD-Z (360-390 bp) (Jensen et al. 2003). We identified optimal amplification conditions by testing a range of $MgCl_2$ concentrations between 1.5 and 3 mM,

gradients of the annealing temperature between 45 and 65°C, and Bovine Serum Albumin (BSA) concentrations up to 0.8 mg/mL.

Once amplification conditions were optimized, PCR reactions were carried out in a final volume of 15 µl containing 1X PCR buffer, 0.2 mM dNTPs, 0.4 µg/µL BSA, 2.5 mM $MgCl_2$, 1U Taq polymerase (ThermoFisher Scientific, Argentina, CAT-10342053), 0.5 µM of each primer, and 1 µL of DNA extracted from blood or 3 µL of DNA from feathers and feces. The amplification conditions were the same for both primer sets consisting on an initial step of 95°C for 3 min, 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 50 s, followed by a final step at 72°C for 5 min. We separated the PCR products through agarose gel electrophoresis and stained them with ethidium bromide.

We successfully amplified CHD from blood, plucked feathers and feces of three specimens of *A. ambiguus,* including a breeding couple whose sex was previously known and that were thus used as controls for this experiment (Fig. 1). The products amplified with the 2550F/2718R primer set yielded a single band (ZZ) in the male (~660 pb) and two discrete bands (ZW) in the female (~660 pb and ~490 pb). A 1.8 % agarose-gel electrophoresis run for 40 minutes at 80 volts was enough to distinguish the two profiles.

In contrast, amplification with P2/P8 failed to show distinct patterns for males and females, even after running 3% agarose gel electrophoresis for six hours. While specific products of the CHD were successfully amplified with these primers, size differences in the CHD-Z and CHD-W products seem to be too small to distinguish sexes using agarose gels. Therefore, polyacrylamide electrophoresis is necessary to recognize male and female profiles using products amplified with P2/P8, which requires

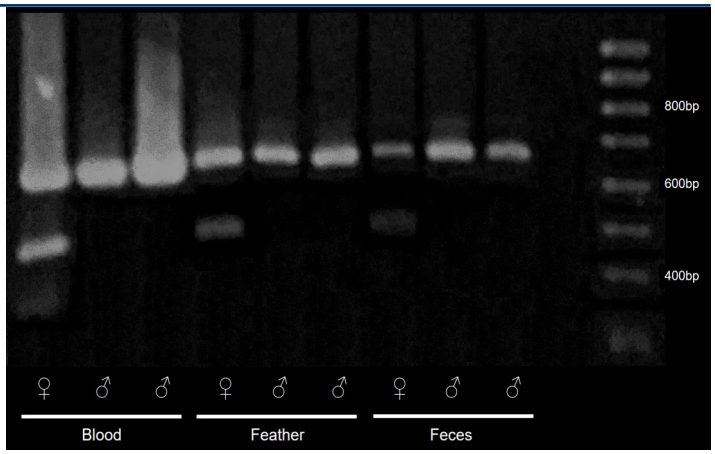

Figure 1. Differential amplification of CHD in one female and two males of *Ara ambiguus* from blood, plucked feathers and fecal samples using primers 2550F/2718R. Amplification from the female yields two well differentiated bands (Z and W), whereas the males show a single band (Z), regardless of the type of sample used to obtain these products. The female and the first male included in this assay correspond to a breeding couple of known sex and were used as controls for all the experiments.

further time and efforts (Griffiths *et al.* 1998, Miyaki *et al.* 1998, Vucicevic *et al.* 2013). Since only the 2550F/2718R primers yielded unique patterns for ZZ and ZW, P2/P8 were excluded from subsequent experiments. The PCR products with the 2550F/2718R primers in the four remaining species of *Ara* and the "harlequin macaw" showed identical patterns in size and specificity to those obtained in *A. ambiguus* (Fig. 2). Overall, 20 out of 33 assessed specimens were males and 13 were females (Table 1).

In the six individuals of *A. ambiguus* for which all three types of tissue were collected (blood, feathers and feces), male and female profiles were clearly discernible. Furthermore, amplification patterns were identical across tissues of the same individual (Fig. 1), but amplicons obtained from fecal DNA displayed less intense bands. Degradation and low concentration of fecal DNA may increase the chance of false homozygous genotypes caused by biased amplification of only one allele during the PCR reaction, usually the shorter PCR product, *i.e.*

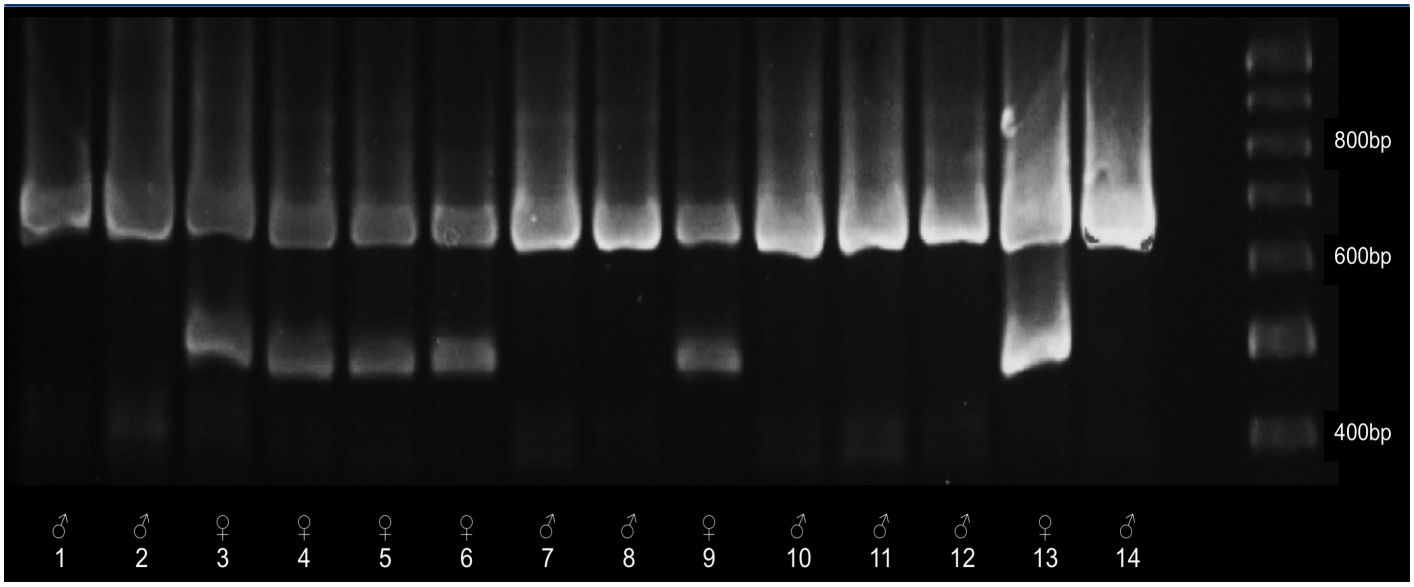

Figure 2. PCR products of CHD in five species of *Ara* using primers 2550F/2718R and DNA extracted from plucked feathers. Lanes 1. *A. ararauna;* 2. *A. chloropterus;* 3. *A. ararauna x A. chloropterus;* 4, 5 and 6. *A. macao;* 7-12. *A. ambiguus. Ara ambiguus* controls appear in lanes 13 (Female) and 14 (Male).

CHD-W. This phenomenon is known as 'allelic dropout' (Robertson & Gemmell 2006, Gebhardt & Waits 2008). In this case, the effect of allelic dropout would translate into exclusive and artefactual amplification of either CHD-Z or CHD-W. However, we found no evidence of this type of error since amplification profiles of control samples matched the expected pattern, all the samples were successfully amplified with no variation among different DNA sources for the same individual, and no homozygous genotypes were observed for the short fragment (CHD-W).

Blood is known to yield high DNA quantities, and it renders the best choice for molecular sexing whenever blood samples need to be collected for other reasons such as measuring chemical or other blood parameters. However, fresh fecal samples are a convenient option to avoid pain, minimize stress and reduce risk of injury. Also, fecal samples can be more easily assigned to a particular animal source than other non-invasive samples such as molted feathers, which is an advantage in field studies. However, preservation of fecal DNA is critical for successful genotyping since its concentration is typically low and it suffers rapid degradation after deposition (Piggott 2004, Panasci 2011). Whenever transportation of fecal samples to the lab within a short time period (<24 h) is unrealistic or impractical, other DNA preservation methods such as freezing, desiccation in silica beads or storage in various buffers may also provide a feasible option (Seutin *et al.* 1991, Taberlet *et al.* 1999). Alternatively, two ventral feathers were enough to obtain reliable genotypes and therefore collection of larger numbers of feathers or prominent feathers such as rectrices is considered unnecessary in these birds.

Our amplification approach of CHD using 2550F/2718R allowed the identification of sexes in all the tested species of *Ara* using feces, plucked feathers or blood as alternative sources of genetic material. Even though this study was not designed to evaluate amplification success, PCR products were obtained from all samples regardless of the DNA source. Our results show that the genetic tool presented here is effective, versatile and can be implemented routinely in any standard molecular biology laboratory to identify the sex in neotropical macaws. Molecular sexing

through non-invasive sampling is particularly useful in controlled habitats such as zoos and animal refuges where minimum stress needs to be caused to identify sexes, a previous condition to establish breeding couples and implement management plans.

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Supplementary Table. Sampled individuals and type of collected tissue.

Sample Code [1]	Known Sex	Species	Inferred Sex	Source [2]
OOO7OAD62O	Hembra	*A. ambiguus*	Female	SZP
OOO7OABOE7	Unknown	*A. ambiguus*	Female	SZP
54594288	Macho	*A. ambiguus*	Male	SZP
97717OOOOO82292	Unknown	*A. ambiguus*	Male	SZP
OOO6FDO83F	Unknown	*A. ambiguus*	Female	SZP
OOO6FD4O9O	Unknown	*A. ambiguus*	Female	SZP
OOO6FD123F	Unknown	*A. ambiguus*	Male	SZP
55325034	Unknown	*A. ambiguus*	Female	SZP
47861884	Unknown	*A. ambiguus*	Male	SZP
977170OOOO56249	Unknown	*A. ambiguus*	Male	SZP
977170OOOO58388	Unknown	*A. ambiguus*	Male	SZP
00O6FCFEO7	Unknown	*A. ambiguus*	Male	SZP
OOO70AB1FA	Unknown	*A. ambiguus*	Female	SZP
OOO6FCFO79	Unknown	*A. ambiguus*	Male	SZP
OOO71OD575O	Unknown	*A. ambiguus*	Male	SZP
97719000006229O	Unknown	*A. ambiguus*	Female	SZP
00070AA69E	Unknown	*A. ambiguus*	Female	SZP
00070D33913	Unknown	*A. ambiguus*	Male	SZP
0006FD081A	Unknown	*A. ambiguus*	Male	SZP
0006FD0F97	Unknown	*A. ambiguus*	Male	SZP
956017000016491	Unknown	*A. ambiguus*	Female	SZP
95017000016818	Unknown	*A. ambiguus*	Male	SZP
0006FCEDA4	Unknown	*A. ambiguus*	Male	SZP
0006CA7E00	Unknown	*A. ambiguus*	Male	SZP
AI 696	Unknown	*A. militaris*	Male	JLPV & HFRG
AI 697	Unknown	*A. militaris*	Male	JLPV & HFRG
AI 364	Unknown	*A. militaris*	Male	JLPV & HFRG
977170000065409	Unknown	*A. ararauna*	Male	SZP
A200G 219	Unknown	*A. chloropterus*	Male	SZP
AZOOG261	Unknown	*A. ararauna* x *A. chloropterus*	Female	SZP
977170000065329	Unknown	*A. macao*	Female	SZP
977170000051305	Unknown	*A. macao*	Female	SZP
AZOOG 115	Unknown	*A. macao*	Female	SZP

1. Microchip (SZP) or individual code. 2. Santa Fe Zoological Park (SZP) or, JLPV & HFRG (Juan Luis Parra-Vergara and Hector Fabio Rivera-Gutiérrez).