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# SEX IDENTIFICATION IN RAPTORS USING PCR

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ABSTRACT.—Recent discovery of a gene on the W-chromosome of birds provides a method for sexing in a variety of avian taxa. We investigated the possible use of polymerase chain reaction (PCR) primers specific to the CHD-W (chromodomain-helicase-DNA-binding on the W-chromosome) gene to identify sex in nine species of raptors. Blood was collected from birds of known-sex (female and male) and DNA was extracted. PCR, using primers P2 and P3, was performed followed by restriction enzyme digestion of the PCR products. Primers P2 and P3, specific to the CHD genes, reliably confirmed the sex in all 38 birds tested representing nine species and four families. A convenient, inexpensive and effective procedure was developed for blood collection, storage and subsequent DNA isolation and PCR analysis.

KEY WORDS: *CHD gene; sex identification; raptors; W-chromosome; PCR.*

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Identificación de sexo en aves rapaces mediante la utilización de la RCP

RESUMEN.—El reciente descubrimiento de un gene en los cromosomas W de las aves es un metodo para sexar una variedad de taxones aviares. Hemos investigado el posible uso de la reacción de la cadena polymersa (RCP) con el fin de identificar el sexo en nueve especies de rapaces. Muestras de sangre fueron obtenidas de seis aves de las cuales se conocía su sexo (machos y hembras), el ADN fue extraido. La RCP fue obtenida mediante la utilización de P2 y P3 y la restricción de la enzima digestiva de los productos de la RCP. Este método permitió, en forma confiable identificar el sexo en las 30 aves, las cuales representaban nueve especies y cuatro familias. Un método conveniente y poco costoso fue desarrollado para la recolección de muestras de sangre, alamacenaje y el subsecuente aislamiento del ADN y el análisis de la RCP.

[Traducción de César Márquez]

The identification of sex is often a problem when studying raptors because sexes are not distinct morphologically. This problem is especially true in juveniles and hatchlings. One effective solution is to exploit DNA markers to diagnose sex. Birds show female heterogamety in having one W- and one Z-sex chromosome whereas males have two Z-chromosomes. Because of this, a simple polymerase chain reaction (PCR) technique can be used to identify sex across a broad range of bird taxa at any stage of development (Griffiths et al. 1996). PCR is a robust technique which can target and amplify specific sequences of DNA (Mullis and Faloona 1987). The PCR primers, P2 and P3, amplify a highly conserved region on the W-sex chromosome known as the CHD (chromodomain-helicase-DNA-binding) gene. A second version of the CHD gene (CHD-NW), not W-linked, is also amplified by the primer pair and is present in both female and male birds. Recently, CHD-NW has been shown to be linked to the Z-chromosome in chickens (Griffiths and Korn 1997). This finding indicates that, among a wide variety of bird species, amplification using the single set of PCR primers followed by restriction digestion of the PCR products will allow for discrimination between the presence of the W-linked CHD gene (unique to female birds) and the CHD-NW gene. A specific restriction enzyme will cut the CHD-NW amplification product but not the W-linked version. The presence/absence of a 110 base pair (bp) band, following enzyme digestion, is diagnostic for sex identification in all bird species previously tested (Griffiths et al. 1996). Our objective for this study was to determine the reliability of PCR primers P2 and P3 in the sexual identification of a variety of raptors.

## MATERIALS AND METHODS

Blood was collected from 38 individuals of known-sex from each of the following species: Bald Eagle (*Haliaeetus leucocephalus*), Red-tailed Hawk (*Buteo jamaicensis*), Red-shouldered Hawk (*Buteo lineatus*), Osprey (*Pandion haliaetus*), American Kestrel (*Falco sparverius*), Merlin (*Falco columbarius*), Black Vulture (*Coragyps atratus*), Barred Owl (*Strix varia*) and Great-horned Owl (*Bubo virginianus*).

The sex of each bird was determined through necropsy, reproductive behavior and/or morphometric and behavioral data. Blood was drawn from the brachial vein of the wing using a 1–3 ml syringe with a 22–25 gauge needle (depending on the species) after swabbing the area with alcohol. Approximately 100 μl of blood was transferred from the syringe to a heparinized microhematocrit tube (Becton Dickinson, Franklin Lakes, NJ U.S.A.) for DNA extraction purposes. Both ends of the tube were plugged with microhematocrit tube sealer (Becton Dickinson). The microhematocrit tube was stored at 4°C for one day to several weeks prior to DNA extraction. If longer storage was required, tubes were placed at −20°C.

DNA was extracted from whole blood using InstaGene Whole Blood Kit (BioRad, Hercules, CA U.S.A.) according to manufacturer's protocol with the following modifications. Five microliters of whole blood per sample were added to a 1.5 ml microcentrifuge tube containing 1 ml of the supplied lysis buffer. The tube was incubated 8–15 min at room temperature and the supernatant discarded. The remaining pellet was washed twice with lysis buffer, carefully removing the supernatant each time. Two hundred microliters of InstaGene matrix was added to the pellet and the tube incubated 8 min at 70°C. The sample was then vortexed, incubated at 95°C for 4 min and centrifuged (15 000 rpm) for 1 min. The resulting supernatant was stored at −20°C per kit instructions until used in PCR analysis.

Standardized PCR reactions were performed twice on DNA from all birds using a 96-well microtiter plate format (Falcon) in a MJ Research Model PTC-100 Programmable DNA Thermal Controller to determine repeatability of PCR reaction conditions. A PCR reaction volume of 20 μl per sample contained: 1× *Taq* DNA polymerase buffer, 200 μM each dNTPs, 3.5 mM $MgCl_2$ primers P2 (5′TCTGCATCGCTAAATCCTTT3′) and P3(5′AGATATTCCGGATCTGATAGTGA3′) (National Biosciences, Inc., Plymouth, MN U.S.A.) at 1 μM each, 100–200 ng of genomic DNA (5 μl of DNA extraction reaction) and 0.5 units of *AmpliTaq* DNA polymerase (Perkin Elmer, Foster City, CA U.S.A.). Cycling parameters were 94°C for 1.5 min, followed by 56°C for 15 sec, 72°C for 15 sec, 94°C for 30 sec, for 30 cycles and one cycle of 56°C for 1 min and 72°C for 5 min. Negative controls containing water were run with every PCR and precautions were taken to avoid contamination (Thomas and Paabo 1993).

Following PCR, all samples were subjected to restriction enzyme digest with *HaeIII*. Five units of *HaeIII* (New England Biolabs, Beverly, CA U.S.A.) were used to cut 7 μl of each PCR reaction following manufacturer's recommendations for appropriate buffer and temperature. Restriction enzyme digestion reaction components were prepared as a master mix to ensure consistent results across all samples.

Samples were electrophoresed on a 2.0% agarose gel (1:1 Amersham Life Sciences, Arlington Heights, IL U.S.A.; Life Technologies, Gaithersburg, MD U.S.A.) at 100 V for 1–2 hr in 1× TBE buffer (90 mM Tris-borate, 2 mM EDTA pH = 8.0) followed by staining for 20 min in 0.5 μg/ml ethidium bromide solution (Maniatis et al. 1982).

## RESULTS

For all species tested in this study, primers P2 and P3 produced PCR products of 110 bp in size in both female and male birds. These primers are specific to both versions of the CHD gene, CHD-W, on the W-chromosome (unique to females) and CHD-NW on a non-W-chromosome (present in both sexes) (Griffiths et al. 1996, Ellegren 1996). This results in the presence of two PCR fragments in females, both 110 bp in size. Males have only one fragment type, CHD-NW. Digestion of the CHD-NW product with a restriction enzyme allows for discrimination between the two PCR products and the determination of sex. The restriction enzyme *HaeIII* was used to cut the CHD-NW product (into two fragments of 45 bp and 65 bp); the CHD-W product remained intact (Fig. 1). For 34 birds, the sex of each was correctly identified by the PCR/*HaeIII* enzyme digest reaction. *HaeIII* did not digest the CHD-NW products for the four Barred Owl samples. This PCR product was subsequently isolated from a known-male sample and sequenced. The sequence data revealed the loss of the *HaeIII* site and provided a candidate *MboII* site for testing. Upon testing in four known-sex samples, *MboII* provided accurate discrimination between female and male samples.

## DISCUSSION

We found that PCR primers P2 and P3 located on the CHD genes of birds reliably sexed the raptors we studied. Primers P2 and P3 confirmed the sex in all 38 species of raptors sampled representing nine species and four families. Other molecular methods have been used to sex raptors but are not useful across taxa and can be a challenge in terms of technique and resources (Longmire et al. 1991, May et al. 1993). PCR is a more straightforward, less labor-intensive technique and more amenable to implementation in less technical settings.

Our study developed a convenient, inexpensive and effective procedure for blood collection, storage and subsequent DNA isolation. Blood is collected in heparinized microhematocrit tubes that can be stored at 4°C for several weeks prior to DNA extraction and still yield viable, high molecular weight DNA. Preparation of DNA from whole blood using a commercial DNA extraction kit expedited the procedure and eliminated the use of hazardous reagents. The PCR protocol works well in a 96-well format allowing for the processing of a large number of samples at one time. Although

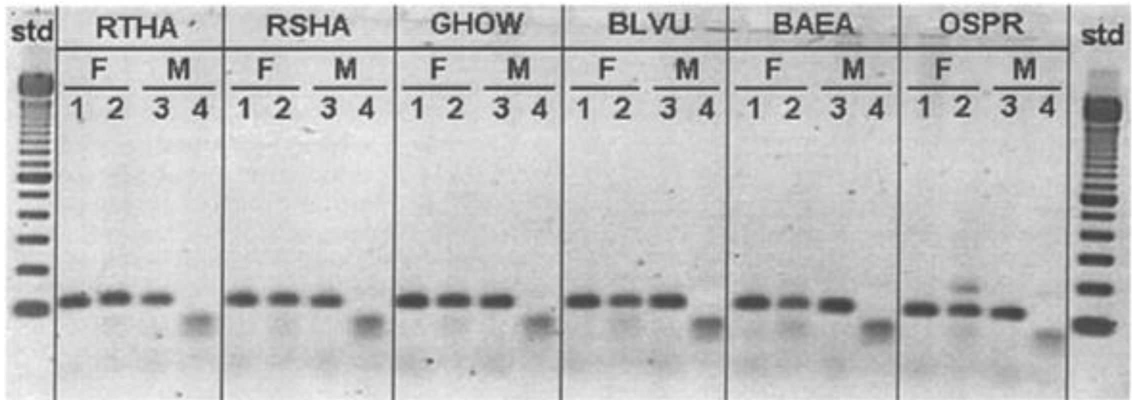


Figure 1. PCR products and corresponding *HaeIII* enzyme digests from one known female and one known male from six of nine raptor species evaluated. For each species (RTHA = Red-tailed Hawk; RSHA = Red-shouldered Hawk; GHOW = Great Horned Owl; BLVU = Black Vulture; BAEA = Bald Eagle; OSPR = Osprey), the first two lanes (1 and 2) represent the one female sample; the next two lanes (3 and 4) represent the one male sample. Lanes 1 and 3 show the 110 bp PCR products generated using primers P2 and P3. Lanes 2 and 4 show the results from the *HaeIII* enzyme digestion. The 'std' lanes contain a 100 bp molecular weight size standard (Life Technologies).

blood was the tissue source for DNA in this study, the sensitivity and specificity of the PCR technology should allow for the use of other tissue sources such as feathers. Based on the fact that sex identification was not shown through the use of a single enzyme (*HaeIII*) for every species tested in this study (Barred owl required *MboII*), known-sex birds from untested species could be initially evaluated using a panel of restriction enzymes. Griffiths et al. (1996) suggested several enzymes that appear to be appropriate over a broad range of avian families and would provide a reasonable starting point. Future modifications of this technique to include primers to the CHD genes that amplify across an intron may eliminate the need for restriction enzymes in some species (Ellegren and Shelton 1997).

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LITERATURE CITED

ELLEGREN, H. 1996. First gene on the avian W-chromosome (CHD) provides a tag for universal sexing of non-ratite birds. *Proc. R. Soc. London Ser. B* 263:1635–1641.

——— AND B. SHELTON. 1997. New tools for sex identification and the study of sex allocation in birds. *Trends Ecol. Evol.* 12:255–259.

GRIFFITHS, R., S. DAAN AND C. DIJKSTRA. 1996. Sex identification in birds using two CHD genes. *Proc. R. Soc. London Ser. B* 263:1251–1256.

——— AND R. KORN. 1997. A CHD1 gene is Z chromosome linked in the chicken *Gallus domesticus*. *Gene* 197: 225–229.

LONGMIRE, J.L., R.E. AMBROSE, N.C. BROWN, T.J. CADE, T.L. MAECHTLE, W.S. SEEGAR, F.P. WARD AND C.M. WHITE. 1991. Pages 217–229 *in* T. Burke, G. Dolf, A.J. Jefferys and W.R. Wolff [EDS.], DNA fingerprinting: approaches and applications. Birkhauser Verlag, Basel, Switzerland.

MANIATIS, T., E.F. FRITCH AND K.J. SAMBROOK. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY U.S.A.

MAY, C.A., J.H. WHETTON AND D.T. PARKIN. 1993. Polymorphic sexspecific sequences in birds of prey. *Proc. Royal Soc. London Ser. B* 253:271–276.

MULLIS, K.B. AND F. FALOONA. 1987. Specific synthesis of DNA in vitro via a polymerased catalyzed chain reaction. *Methods of Enzymology* 155:335–350.

THOMAS, W.K. AND S. PAABO. 1993. DNA sequences from old tissue remains. *Methods of Enzymology* 224:406–419.

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