Rapid and Low-Cost Molecular Sexing of a Corvid Songbird Using a Single Protocol with Two Universal Primer Sets

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Source: Ardeola, 68(2) : 501-510

Published By: Spanish Society of Ornithology

URL: https://doi.org/10.13157/arla.68.2.2021.sc1

Ardeola 68(2), 2021, 501-510 DOI: 10.13157/arla.68.2.2021.sc1

Short Communications

RAPID AND LOW-COST MOLECULAR SEXING OF A CORVID SONGBIRD USING A SINGLE PROTOCOL WITH TWO UNIVERSAL PRIMER SETS

SEXADO MOLECULAR RÁPIDO Y ECONÓMICO PARA UN CÓRVIDO, UTILIZANDO UN MISMO PROTOCOLO CON DOS PARES DE CEBADORES UNIVERSALES

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SUMMARY.—The absence of sex dimorphism in many bird species complicates sex determination by direct observation, hindering sex-specific studies. Standard protocols for molecular sexing include polymerase chain reaction (PCR) amplification of intron regions of the Chromodomain-Helicase DNA-binding protein 1 (*CHD1*) gene. While several methods have been studied, their usefulness for songbirds (Passeriformes) has not been consistent and has largely depended on target species and on time-consuming primer-set specific optimisation of available protocols. We tested a molecular sexing protocol with two universal primer sets (P2/P8 and 2550F/2718R) in a corvid songbird: the Plush-crested Jay *Cyanocorax chrysops*. The protocol was rapid and inexpensive as well as highly effective. Using 2550F/2718R, females were revealed by two bands separated for some 200 base pairs (bp) that resolved easily on 0.8% agarose gel. Conversely, P2/P8 female amplicons differed in roughly 30 bp and a more expensive 3% agarose gel was necessary to reveal them. Our results are contextualised with an up-to-

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date literature survey of molecular sexing in other corvids. The primer set 2550F/2718R is found to be effective, providing a reliable and low-cost method for sexing jays and other corvids. —Lois-Milevicich, J., Gómez, R.O., Ursino, C.A., Lois, N.A. & de la Colina, A. (2021). Rapid and low-cost molecular sexing of a corvid songbird using a single protocol with two universal primer sets. *Ardeola*, 68: 501-510.

Key words: 2550F/2718R, CHD1 gene, Corvidae, P2/P8, Passeriformes.

RESUMEN.—La ausencia de dimorfismo sexual en muchas especies de aves dificulta la determinación del sexo a simple vista, lo cual complejiza los estudios sexo-específicos que dependen de esta determinación. Los protocolos estándar para sexado molecular incluyen una amplificación por reacción en cadena de la polimerasa (PCR) de intrones del gen de la helicasa con cromodominio de unión a ADN 1 (CHD1). Pese a que muchos métodos han sido estudiados, su utilidad en aves cantoras (Passeriformes) no ha sido consistente y su optimización ha dependido de combinar protocolos disponibles y cebadores determinados de manera específica para cada especie. En este trabajo testamos un protocolo para sexado molecular en urracas (chara moñuda *Cyanocorax chrysops*) usando dos pares de cebadores universales (P2/P8 y 2550F/2718R). El protocolo resultó rápido, económico y altamente efectivo. Las hembras fueron diferenciadas de los machos a través de la visualización de dos bandas separadas por 200 pares de bases (pb) aproximadamente usando el par 2550F/2718R, las cuales fueron fácilmente reveladas en un gel de agarosa al 0,8%. En cambio, los fragmentos obtenidos para las hembras usando el par P2/P8 difirieron en apenas 30 pb y necesitaron un gel de agarosa más concentrado (3%) para diferenciarse. Nuestros resultados se contextualizan con una revisión de la literatura actualizada sobre el sexado molecular en córvidos. El par de cebadores 2550F/2718R resulta efectivo, proporcionando un método confiable y de bajo costo para determinar el sexo de urracas y otros córvidos. —Lois-Milevicich, J., Gómez, R.O., Ursino, C.A., Lois, N.A. y de la Colina, A. (2021). Sexado molecular rápido y económico para un córvido, utilizando un mismo protocolo con dos pares de cebadores universales. *Ardeola*, 68: 501-510.

Palabras clave: 2550F/2718R, Corvidae, gen CHD1, P2/P8, Passeriformes.

INTRODUCTION

Sex identification in birds is often a challenge due to the lack or late development of evident sex dimorphism, which may constitute a major drawback in many ecological and evolutionary studies as well as in *ex situ* conservation management. Given that more than half of avian species are sexually monomorphic as adults (Price & Birch, 1996) and that sexing based on surgical procedures is neither possible nor ethically acceptable in most cases, minimally invasive molecular techniques targeting sex-linked genes have been developed as a fast and reliable alternative for sex determination (Griffiths *et al*., 1996; Fridolfsson & Ellegren, 1999; Morinha *et al*., 2013, 2015; Dawson *et al*., 2016; Liang *et al*., 2019).

Several sexing assays that rely on polymerase chain reaction (PCR) amplification of intron regions within the highly conserved Chromodomain-Helicase DNA-binding protein 1 (*CHD1*) gene have been advanced to date (e.g., Griffiths *et al*., 1996, 1998; Kahn *et al*., 1998; Fridolfsson & Ellegren, 1999; Ito *et al*., 2003; Mucci *et al*., 2017), with those based on the universal primer sets P2/P8 (Griffiths *et al*., 1998) and 2550F/2718R (Fridolfsson & Ellegren, 1999) among the most extensively used (Ong & Vellayan, 2008; Kocijan *et al*., 2011; Khaerunnisa *et al*., 2013; Vucicevic *et al*., 2013; Çakmak *et al*., 2017; Mataragka *et al*., 2020). These methods exploit the different amplicon size from *CHD1-Z* and *CHD1-W*, yielding one band for males (homogametic, *ZZ*) and two bands for females (heterogametic, *ZW*) on

standard agarose gel electrophoresis (Griffiths *et al*., 1998; Fridolfsson & Ellegren, 1999), and have proved to be relatively effective across most non-ratite avian orders (Griffiths *et al*., 1998; Fridolfsson & Ellegren, 1999; Vucicevic *et al*., 2013; Çakmak *et al*., 2017; Ágh *et al*., 2018; Rodrigues *et al*., 2019; Mataragka *et al*., 2020; and references cited therein). However, their usefulness among songbirds (Passeriformes) has not been consistent and has largely depended on species-specific time-consuming optimisation of available protocols (Kocijan *et al*., 2011; Vucicevic *et al*., 2013; Dawson *et al*., 2016; Çakmak *et al*., 2017). In particular, the primer set P2/P8 produces very similar amplicons (in size) which makes it difficult to successfully reveal and identify the sexes (Dawson *et al*., 2001). This problem can be solved using more expensive revealing methods such as a more concentrated agarose gel or even an acrylamide gel (Griesser & Ekman, 2005; Dawson *et al*., 2001; Vucicevic *et al*., 2013; Ágh *et al*., 2018). On the other hand, the primer set 2550F/2718R produces amplicons that differ more in size, yet in some studies on songbirds, sex discrimination was impossible in more than 95% of the species studied due to the lack of the *W*-allele amplification in the females (Kocijan *et al*., 2011; Çakmak *et al*., 2017).

The Plush-crested Jay *Cyanocorax chrysops* is a conspicuous and charismatic songbird of the family Corvidae (crows, jays and allies) (Madge & Burn, 2001). This family includes 129 species in 23 genera currently recognised (Clements *et al*., 2019). As in other corvids (e.g., Bedrosian *et al*., 2008), Plush-crested Jays are sexually monomorphic, hindering sex-specific studies. In this work, we test a single fast and inexpensive protocol for sexing Plush-crested Jays using two different universal primer sets and we discuss our results in the context of an up-to-date literature survey of molecular sexing in corvids.

Methods

Blood samples (0.2ml) were taken by right jugular or metatarsus venipuncture from nine captive Plush-crested Jays (8 adults, 1 juvenile) at the Fundación Temaikèn Zoo, Escobar, Argentina (permit number 66/2018, Dirección de Flora y Fauna, Buenos Aires Province), and stored at room temperature in 0.7ml lysis buffer (100 mM Tris, pH 8.0; 100 mM EDTA, pH 8.0; 10 mM NaCl; 2% SDS). Genomic DNA was extracted from all samples using a standard salting-out protocol (Miller *et al*., 1988). Incubation was carried out in 285µl TNES buffer (50 mM Tris, pH 7.5; 400 mM NaCl; 20 mM EDTA, pH 7.5) with 5µl protease K (10mg/ml) and 15µl 10% SDS at 65ºC for at least 2 h, vortexing each 30 min. Then, 85µl NaCl (5 M) were added to each sample, followed by vortexing for 15s. Samples were centrifuged at 14,000×G for 5 min. Ice-cold isopropyl alcohol was added to the supernatant and, after several inversions, tubes were centrifuged at 14,000×G for 5 min. Isopropyl alcohol was discarded and ice-cold 70% ethanol added to each sample before centrifuging them again at 14,000×G for 5 min (tubes in inverted position with respect to the previous centrifugation). The ethanol was discarded, allowing residual ethanol to evaporate for 30 min, and the pellet resuspended in 50-200µl of ultrapure water, depending on the amount of pellet. The resulting extractions were stored at −20°C. Quality of genomic DNA was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and 0.8% agarose gel electrophoresis.

In order to sex birds, the universal primer set 2550F/2718R (5'-GTTACTGATTCGTC TACGAGA-3'/5'-ATTGAAATGATCCAGT GCTTG-3') of Fridolfsson & Ellegren (1999) was used to amplify intron fragments of the *CHD1* gene. The primer set P2/P8 (5'-TCT GCATCGCTAAATCCTTT-3'/5'-CTCCC AAGGATGAGRAAYTG-3') of Griffiths *et*

al. (1998) was also used for comparative purposes. Each individual was sexed at least seven times, totalling 64 trials (53 with 2550F/2718R and 11 with P2/P8). All PCR reactions were performed in 10µl volumes on a LabNet MultiGene gradient thermal cycler (LabNet International, USA) using ca. 32-232 ng of DNA template, 0.25 units of *Taq* DNA polymerase (Genbiotech, Argentina), 1×PCR reaction buffer, 2.5 mM $MgCl_2$, 0.5 µM of each primer, and 0.1 mM dNTP mix. The thermal profile consisted of an initial denaturation step at 94°C for 4 min, followed by a touch-down scheme of 8 cycles at 94°C for 30s, annealing for 45s (starting at 55°C and decreasing 1°C per cycle until reaching 48°C), 72°C for 45s; then 30 cycles of 94°C for 30s, 48°C for 45s, 72°C for 45s and a final extension step at 74°C for 5 min. The PCR products were separated by electrophoresis in 0.8-3% agarose (Genbiotech, Argentina) gels, run in 1×TAE buffer (40 mM Tris, pH 8.0 –20 mM acetic acid– 1 mM EDTA, pH 8.0) at 90 V during 15-30 min for 2550F/2718R and 40-50 min for P2/P8, stained with fluorescent nucleic acid dye (GelRed® Biotium, Hayward, CA, USA), and visualised in a UV transilluminator (LabNet International, Inc. Edison, NJ, USA; UV light source wavelength 302 nm).

All research presented in the manuscript was conducted following relevant Argentinean regulations (Law of Conservation of Wild Fauna). Captures, handling, and blood sampling were carried out exclusively by a team of professionals from the Fundación Temaikèn Zoo. The protocols used in this study have been subjected to an ethical review process by the Research Ethics Committee of Fundación Temaikèn. This foundation is a member of ALPZA (Asociación Latinoamericana de Parques Zoológicos y Acuarios), WAZA (World Association of Zoos and Aquariums) and AZA (Association of Zoos and Aquariums), and thus complies with their standards on animal welfare.

Results

Sex identification with the primer set 2550F/2718R was successful in all cases, reaching a success rate of 96% (51/53 trials), consistently revealing two females and seven males among the nine jays (Figure 1a). Males were characterized by a single band of ca. 650 base pairs (bp) corresponding to the amplicon from *CHD1-Z*, whereas females were revealed by two bands of ca. 650 bp (*Z* allele) and ca. 450 bp (*W* allele), which were resolved on 0.8, 1, 1.5, 2, and 3% agarose gels and with short electrophoresis times (15-30 min). Note that less concentrated gels (0.8%) needed more extensive times (30 min) than more concentrated gels (2-3%) which were resolved in only 15 min, while gels of intermediate concentration (1-1.5%) needed around 20-25 min. Sex identification using the primer pair P2/P8 had a success rate of 91% (10/11 trials), matching the results of 2550F/2718R, giving in all cases a single band for males of ca. 360 bp (*Z* allele) and two bands for females of ca. 360 bp (*Z* allele) and ca. 390 bp (*W* allele), which were well resolved on 3% agarose gels with running times longer than 40 min (Figure 1b) but not on 0.8-2% agarose gels nor with shorter times, because of the small size-difference between amplicons, and, in some cases, the longer amplicon (*W* allele) was hardly detectable.

Discussion

The low-cost protocol used here, using small quantities of *Taq* DNA polymerase and low-density agarose gels, has proved to be highly effective in sexing a corvid songbird. Although both primer sets are suitable for Plush-crested Jays, this protocol gives better results with primer set 2550F/2718R than with P2/P8, which somewhat agrees with previous studies on diverse avian orders (Vucicevic *et al*., 2013; Çakmak *et al*., 2017; Ágh *et*

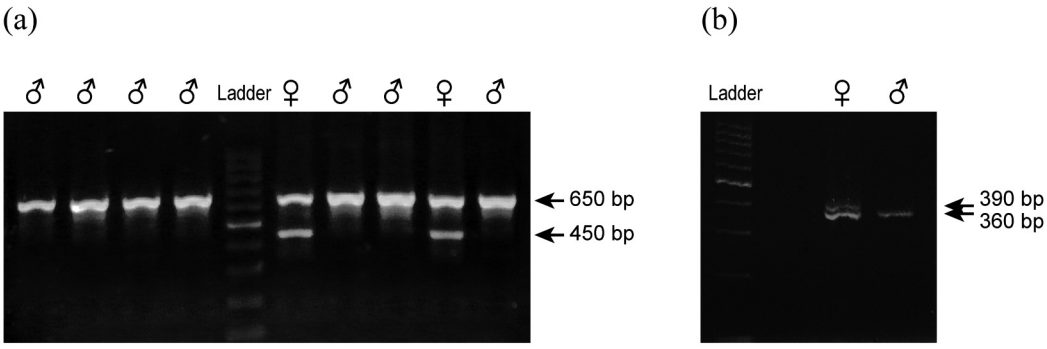

FIG. 1.—Sex determination in Plush-crested Jay using the primer sets: (a) 2550F/2718R in 1% agarose gel after 30 min, and (b) P2/P8 in 3% agarose gel after 45 min. Note that even using a lower agarose concentration and a shorter running time, a greater band separation is achieved using primer set 2550F/2718R.

[Determinación del sexo de urracas utilizando los pares de cebadores: (a) 2550F/2718R en gel de agarosa al 1% tras de 30 min de corrida y (b) P2/P8 en gel de agarosa al 3% después de 45 min de corrida. Nótese que, incluso usando una menor concentración de agarosa y menor tiempo de corrida, se logra una mejor separación de bandas usando el par de cebadores 2550F/2718R.]

al., 2018; but see Mataragka *et al*., 2020). It is noteworthy that using 2550F/2718R the *CHD1-W* amplicon is much smaller than the *CHD1-Z* amplicon and it is often amplified preferentially, which makes the band from *Z* barely distinguishable in females. However, females are easily identified by the different size of the *Z* and *W* amplicons. Conversely, P2/P8 produces a *W* amplicon larger than that of *Z*, which could lead to identifying females as males when preferential amplification occurs (Dawson *et al*., 2001). In addition, *Z* and *W* amplicons differ in roughly 200 bp with 2550F/2718R and can be easily resolved in 0.8% agarose gels with short running times, whereas with P2/P8 the difference between amplicons abridges to less than 50 bp and needs denser agarose or acrylamide gels and longer electrophoresis times for them to be resolved (Dawson *et al*., 2001; Vucicevic *et al*., 2013; Ágh *et al*., 2018; see below).

The efficacy of available protocols based on these primer sets has been irregular among the Passeriformes analysed to date, failing to distinguish between sexes or even to amplify *CHD1* products in many songbird species (Kocijan *et al*., 2011; Vucicevic *et al*., 2013; Dawson *et al*., 2016; Çakmak *et al*., 2017). Only a small fraction of the vast songbird diversity, which comprises over 6,000 species and nearly 60% of extant avian diversity (Dickinson & Christidis, 2014), has been sexed with molecular methods so far, and even fewer with 2550F/2718R (Vucicevic *et al*., 2013; Çakmak *et al*., 2017; Mataragka *et al*., 2020).

However, our literature survey of molecular sexing in Corvidae, which covers 24 studies that encompasses 20 species in 12 genera reveals that these primer sets have produced reliable results consistently in all species analysed so far (Table 1). Each of the 20 species previously analysed has been successfully sexed with either P2/P8 or 2550F/2718R in at least one study. A single study has failed in sexing with P2/P8, with females identified as males (Vucicevic *et al*., 2013), whereas sexing with 2550F/2718R has always been successful. The primer set P2/P8 produces bands of 330-373 bp (*Z* allele)

TABLE 1

Sex determination in Corvidae using primer sets P2/P8 and 2550F/2718R. Successful determinations were depicted as (+). When available, amplicon length is provided in base pair (bp).
[Determinación del sexo de córvidos usando los pares de cebadores P2/P8 y 2550F/2718R. Las determinaciones exitosas se señalan con (+). En los casos reportados, se detalla el largo del fragmento de ADN en pares de bases (bp).]

Species	P2/P8 *Z*/*W* (bp)	2550F/2718R *Z*/*W* (bp)	Observations	References
California Scrub-Jay *Aphelocoma californica*	(+)			Rensel *et al*., 2015
Florida Scrub-Jay *Aphelocoma coerulescens*		(+)		Townsend *et al*., 2011
Transvolcanic jay *Aphelocoma ultramarina*	(+)			Robertson & Gemmell, 2006
Black-throated Magpie-Jay *Calocitta colliei*	(+)	(+)		Mataragka *et al*., 2020
White-throated Magpie-Jay *Calocitta formosa*	(+)			Berg, 2005
American Crow *Corvus brachyrhynchos*		(+)		Yorzinski *et al*., 2006
Common Raven *Corvus corax*	(+)[a]	650/450-400		Fridolfsson & Ellegren, 1999; Stevanov-Pavlovic *et al*., 2013; Vucicevic *et al*., 2013; Gábor *et al*., 2014
Carrion Crow *Corvus corone*	(+)	661/465		Baglione *et al*., 2002; Fukui *et al*., 2008
Rook *Corvus frugilegus*	355/391[a]	650/400	P2/P8 in acrylamide	Dawson, 2005; Stevanov-Pavlovic *et al*., 2013; Vucicevic *et al*., 2013
Hawaiian Crow *Corvus hawaiiensis*	330/380			Jensen *et al*., 2003
Large-billed Crow *Corvus macrorhynchos*		661/465		Fukui *et al*., 2008
Eurasian Jackdaw *Corvus monedula*	(+)			Gábor *et al*., 2014
Steller's Jay *Cyanocitta stelleri*	(+)			Overeem, 2013
Plush-crested Jay *Cyanocorax chrysops*	360/390	650/450		This study

[a] Females, as males, gave a single band of equal length in Vucicevic *et al*. (2013).

TABLE 1 (cont.)

Species	P2/P8 Z/W (bp)	2550F/2718R Z/W (bp)	Observations	References
Azure-winged Magpie *Cyanopica cyanus*	(+)	(+)	P2/P8 in acrylamide	Griesser & Ekman, 2005; Gao *et al*., 2018
Eurasian Jay *Garrulus glandarius*	(+)	673-700/463-500		Çakmak *et al*., 2017; Ágh *et al*., 2018
Eurasian Nutcracker *Nucifraga caryocatactes*	350/390			Jensen *et al*., 2003
Siberian Jay *Perisoreus infaustus*	(+)	(+)		Griesser & Ekman, 2005
Eurasian Magpie *Pica pica*	(+)		P2/P8 in acrylamide	Griesser & Ekman, 2005; Lee *et al*., 2010; Dawson *et al*., 2016
Red-billed Chough *Pyrrhocorax pyrrhocorax*	373/382	(+)		Morinha *et al*., 2013; Trask *et al*., 2016
Taiwan Blue-Magpie *Urocissa caerulea*	309/350	(+)		Wang *et al*., 2007; Lee *et al*., 2010

and 380-391 bp (*W* allele) in most cases, whereas amplicons produced using 2550F/2718R are of 650-700 bp (*Z* allele) and 400-500 bp (*W* allele). Among corvids, the primer set P2/P8 has produced *Z* amplicons 10-50 bp shorter than the corresponding *W* amplicons, which often needed acrylamide gels to be adequately resolved (Griesser & Ekman, 2005), whereas with 2550F/2718R the products from *Z* have resulted 200-250 bp longer than those from *W* and were easily resolved in low-density standard agarose gels.

It is noteworthy that despite the success of these two primer sets, a unique protocol for both was still lacking. We therefore designed a protocol that has been proved to be effective using both primer sets in the Plush-crested Jay, which also represents the first South American corvid successfully sexed by molecular methods.

All in all, our optimized assay based on 2550F/2718R gives the most reliable results and constitutes a rapid, inexpensive, and efficient method for sexing jays. We believe this protocol will result advantageous, especially in studies where sex determination is required and time and resources are critically limited, as is often the case in many conservation projects. This primer set has been useful in successfully sexing other corvids as well, but its effectiveness in other Passeriformes remains a contentious issue (Çakmak *et al*., 2017; Mataragka *et al*., 2020). Further work is needed to determine whether the success of this technique among corvids could extend to other songbirds.

ACKNOWLEDGEMENTS.—JLM, NAL are supported by fellowships from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). ROG, CAU are Research Fellow of UBA-CONICET. Funds from Universidad de Buenos Aires (UBACyT 20020170100521BA) supported this work. We are grateful to Fundación

Temaikèn's staff and particularly to zookeepers from 'El Lugar de las Aves' and vets for providing the blood samples. We would like to thank editors and reviewers for their constructive suggestions. Thanks are extended to W. Grillo for his help to improve the draft writing. We also thank B. Mahler, M. Atencio and J.M. Rojas Ripari for helping in the molecular assays, J.C. Reboreda for continuous support, and V. Confalonieri and I. Miniani for access to and assistance with the NanoDrop.

AUTHOR CONTRIBUTIONS.—Conceptualisation: JL, ROG; Methodology: JL, CAU, NAL; Blood samples accessibility: AC; Writing – original draft preparation: ROG; Writing – review and editing: JL, ROG, CAU, NAL, AC.

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Submitted: January 08, 2021
Minor revision: February 09, 2021
Second version arrived: February 20, 2021
Accepted: March 04, 2021

Editor: Alice Cibois