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PCR PROTOCOLS FOR MOLECULAR SEXING IN MONOMORPHIC BIRDS

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Abstract

More than a half of bird species around the world are monomorphic, therefore they do not show distinct sexual dimorphic traits. The paper aims to present three molecular methods for sex identification of monomorphic companion birds. Samples of feathers, oral swabs and blood were collected from Psittaciformes (Ara ararauna, Psittacus erithacus and Psittacula krameri) and Columbiformes (Columba livia domestica). All samples were tested by three different PCR protocols in order to identify the chromo-helicase-DNA-binding (CHD1)-W gene and CHD1-Z genes in females and the CHD1-Z gene in males. Two protocols were conventional PCR, using P2 and P8, respectively P2 and NP primers, while the third protocol was a multiplex PCR using P0, P2 and P8 primers. As a conclusion, all three PCR protocols can be used for molecular sexing of monomorphic Psittaciformes and Columbiformes companion birds. Feather, oral swab and blood samples provided adequate DNA templates for the sex identification of birds.

Key words*: monomorphic birds, feathers, oral swab, blood, PCR.*

INTRODUCTION

Over half of all bird species globally lack distinct sexual dimorphism (Griffiths et al., 1998; O'Malley, 2005). Among the most popular pet birds are the *Psittaciformes*, commonly known as parrots, which consist of roughly 400 species (Curro, 1998; Forshaw, 2010). Most parrots, including the African Gray Parrot (*Psittacus erithacus*) and the Yellow-breasted Macaw (*Ara ararauna*), are sexually monomorphic (Forshaw, 2010). However, certain parrot species display sexual dimorphism after they reach sexual maturity. For instance, male parakeets (*Melopsittacus undulatus*) typically have blue ceres, whereas females have pinkish-brown ceres. Unfortunately, these sexually dimorphic traits only become apparent after the birds have reached sexual maturity (Forshaw, 2010; O'Malley, 2005). In Rose Ringed Parakeets (*Psittacula krameri*) only adult males display a black neck ring, while females and both sexes' sexually immature birds lack one. The Rose-Ringed Parakeet fails to exhibit sexual dimorphism until reaches the age of 3 years, when it achieves sexual maturity (Forshaw, 2010; O'Malley, 2005).

Columbiformes are considered monogamous. Domestic Pigeons do not exhibit sexual dimorphism, but can sometimes be sexed using traditional sexing techniques. Traditional methods of pigeon sexing involve observing of the characteristic secondary sex features, such as male neck plumage, bird size and head shape (males are larger and have larger beaks) and observing behaviour (the male's characteristic song and dance during the ritual of mating and ponta, a late method, but which has 100% accuracy in females) (Tudor, 1991; O'Malley, 2005).

The male birds are homogametic (ZZ), while the females have two different sex chromosomes (ZW). The amplification of CHD1 (chromo-helicase-DNA binding protein) gene, located in both sex chromosomes of all birds, allowed the sex identification in most avian species (Griffiths et al., 1998). More precisely, sex identification is achieved by amplifying the homologous sections of the two genes CHD1-Z and CHD1-W, including introns, which normally vary in size.

There were described three molecular sexing methods for identification of the two genes: PCR-RFLP (restriction fragment length polymorphism) (Griffiths and Tiwari, 1995; Griffiths et al., 1996), PCR-SSCP (single-strand conformational polymorphism) (Ellegren, 1996; Cortés et al., 1999) and conventional PCR based on intronic size variation (Griffiths et al., 1998). The last one is more rapid and simple, but is not a universal method.

PCR-based bird sexing methods have broad applications in studying reproductive and conservation biology, ecology, and evolution (Morinha et al., 2012). Given that pet birds are highly social and require pairing, molecular sexing is critical in ensuring their early and appropriate welfare needs (Peng and Broom, 2021).

MATERIALS AND METHODS

Sample collection

Samples of contour feathers with intact calamus, oral swabs and blood (dried blood spots) were collected from 4 individuals from order *Psittaciformes* as followed: two Yellow-breasted Macaws (*Ara ararauna*), one African Gray Parrot (*Psittacus erithacus*) and one Rose Ringed Parakeet (*Psittacula krameri*) and from four Domestic Pigeons *(Columba livia domestica)*. Oral swabs were collected using sterile cotton swabs (Prima, Taizhou Honod Medical Co., Ltd., Zhejiang, China). One drop of blood from each bird was collected on filter paper (dried blood spot).

The samples were collected with the consent of the owners, who voluntarily participated in the study. All samples were labelled individually and stored at -20°C until processing.

DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from all samples of feathers (n = 8), oral swabs (n = 8) and dried blood spots (n = 8) collected from birds. The calamus of each feather was sectioned in small pieces (2-3 mm) with the help of a sterile scalpel and then was subjected to mechanical destruction by high-speed shaking together with steel beads, using TissueLyserII (Qiagen, US). Oral swabs were transferred to 1.5 ml Eppendorf tubes using sterile scissors. The dried blood spots were subjected to DNA extraction. DNA extraction was performed using a commercial kit (Isolate II Genomic DNA kit; Meridian Bioscience, USA) following the manufacturer`s protocol. For all types of tissues, the same protocol was used.

All the samples were tested for the presence of specific genes CHD1-W and CHD1-Z by three PCR protocols. PCR was carried out in a 25 µl reaction mixture consisting of 12.5 µl of MyTaq Red HS Mix (Meridian Bioscience, USA) and 25 pM of each primer. The volume of DNA template was 4 µl.

Protocol 1. The identification of the CHD1 gene was performed according to the protocol described by Griffiths et al. (1998), using the primers P2 (5′-TCT GCA TCG CTA AAT CCT TT-3′) and P8 (5′-CTC CCA AGG ATG AGR AAY TG-3′) (Generi-Biotech, Hradec Králove, Czech Republic). Cycling conditions were: 95°C for 5 min for initial denaturation, followed by 48°C for 45 sec, 72°C for 45 sec and 95°C for 30 sec (35 cycles); 48°C for 1 min and final extension at 72°C for 5 min.

Protocol 2. The identification of the CHD1 gene was performed according to the protocol described by Ito et al. (2003), using the primers P2 (5′-TCT GCA TCG CTA AAT CCT TT-3′) and NP (5'-GAG AAA CTG TGC AAA ACAG-3') (Generi-Biotech, Hradec Králove, Czech Republic). Cycling conditions were: 95°C for 5 min for initial denaturation, followed by 52°C for 45 sec, 72°C for 45 sec and 95°C for 30 sec (35 cycles); 50°C for 1 min and final extension at 72°C for 5 min.

Protocol 3. The identification of the CHD1 gene was performed according to the protocol described by Han et al. (2009), using the primers P0 (5′-ATT GAG TTG GAA CCA GAI CA-3′), P2 (5′-TCT GCA TCG CTA AAT CCT TT-3′) and P8 (5′-CTC CCA AGG ATG AGR AAY TG-3′) (Generi-Biotech, Hradec Králove, Czech Republic). Cycling conditions were: 95°C for 5 min for initial denaturation, followed by 95°C for 30 sec, 53°C for 30 sec and 72°C for 45 sec (35 cycles) and 72°C for 5 min for final extension.

For all the three protocols the amplification was performed in Bio-Rad C1000TM Thermal Cycler (Bio-Rad Laboratories, Hercules,

California). Aliquots of each PCR product were electrophoresed on 3% agarose gel stained with RedSafe Nucleic Acid Staining Solution 20.000x (iNtRON Biotechnology), and examined for the presence of the specific fragment under UV light (Bio-Rad BioDoc-ItTM Imagine System). DNA fragment size was compared with a standard molecular weight, 100 bp DNA ladder (Fermentas; Thermo Fisher Scientific, Waltham, Massachusetts). Females are characterized by obtaining two bands corresponding to the CHD1W and CHD1Z genes, while males present only one band corresponding to the CHD1Z gene.

RESULTS AND DISCUSSIONS

All three PCR protocols tested allowed sex determination in both, *Psittaciformes* and *Columbiformes* bird species. The results of molecular sexing of monomorphic companion birds from oral swabs, feathers and dried blood spot are presented in Table 1.

Table 1. Results of molecular sexing monomorphic companion birds included in this study

Birds	Protocol 1	Protocol 2	Protocol 3
Psittaciformes			
Yellow-breasted Macaw 1 (*Ara ararauna*)	M	M	M
Yellow-breasted Macaw 2 (*Ara ararauna*)	M	M	M
African Gray Parrot (*Psittacus erithacus*)	M	M	M
Rose Ringed Parakeet (*Psittacula krameri*)	F	F	F
Columbiformes			
Pigeon 1 (*Columba livia domestica*)	M	M	M
Pigeon 2 (*Columba livia domestica*)	M	M	M
Pigeon 3 (*Columba livia domestica*)	M	M	M
Pigeon 4 (*Columba livia domestica*)	F	F	F

The PCR products showed two bands in females (corresponding to CHD1-W and CHD1-Z genes) and a single band in male (corresponding to CHD1-Z gene). The PCR electrophoresis gel for sex identification in *Columbiformes* using protocol 2 was presented in the Figure 1.

All three tested PCR protocols targeted to identify the CHD1, gene that has been conserved in the avian W chromosome and which allows sex identification in most species.

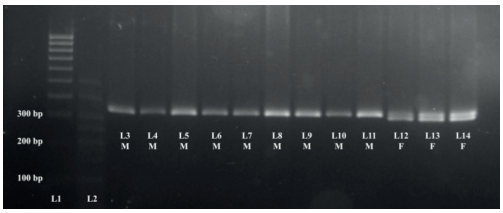

Figure 1. PCR gel from *Columbiformes* using protocol 2. Legend: L1 - size standard (100-bp DNA ladder); L2 - size standard (25-bp DNA ladder); L3, L6, L9, L12 - oral swab; L4, L7, L10, L13 - feathers; L5, L8, L11, L14 - dried blood spots; M - male; F - female

The most used and cited method of molecular sexing of birds is the P2/P8 sexing test described by Griffiths et al. (1998). The test involves a simple conventional PCR performed with a single pair of primers to amplify an intron in CHD-W and CHD-Z genes. The result shows a band in males and two bands in females. The P2/P8 test was successful used to sex identification in 27 bird species from the class Aves. *Psittaciformes* and *Columbiformes* birds species can be can be sexed by this molecular method (Turcu et al., 2022; Turcu et al., 2023). Because is based on intronic length polymorphism size variation, the method is not universal and cannot be used for sex identification in birds that not present or have a small size variation (Han et al., 2009).

The second protocol presented here was described by Ito et al. (2003) and it is used for DNA sexing. This method is based on the intronic length variation between CHD1-W and CHD1-Z. This method was developed for sex identification in *Falconiformes*, but in the present paper we demonstrated that it can be use with success for molecular sex identification in *Psittaciformes* and *Columbiformes.* Our results showed that P2/NP can offer the strong bands in both tested species.

Han et al. (2009) described a multiplex PCR for DNA sexing in birds. In addition to P2/P8 primer pair described by Griffiths et al (2009), a new P0 primer is used. P0 is a specific primer that can amplify a sequence present only on CHD1-W gene. The advantage of the multiplex PCR method is that it can determine the sex of the birds regardless of the intron size variation. In some species, the females can display three different bands, the extra-band being amplified by the primers P0/P2. In the present paper we

have identified an extra band in the female Rose Ringed Parakeet (*Psittacula krameri*) (Figure 2).

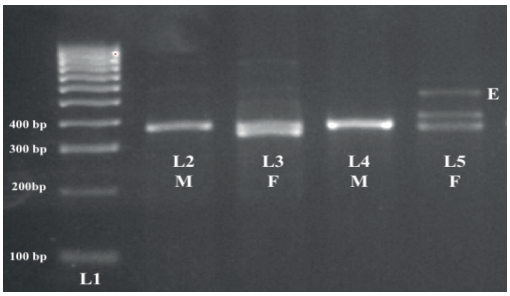

Figure 2. PCR gel from *Columbiformes* and *Psittaciformes* using protocol 3.
Legend: L1 - size standard (100-bp DNA ladder); L2, L3 - *Columba livia domestica*; L4; L5 - *Psittacula krameri*; M - male; F - female; E - additional band

CONCLUSIONS

Feather, oral swab and dried blood spot samples provided DNA templates suitable for sexing the monomorphic birds. All three PCR protocols can be used for molecular sexing of the following birds orders: *Psittaciformes* and *Columbiformes*. We recommend protocol which uses the P2/NP primers pair, described by Ito et al. (2003), which gives the strongest bands in both tested species.

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