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*Research Article*

# ISOLATION OF DNA BY PHENOL-CHLOROFORM EXTRACTION METHOD FROM SHED AND WAITING FEATHERS OF *PSITTACIFORMES* BIRD ORDER

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**ABSTRACT.** In this study, it was aimed to isolate DNA from shed and waiting (at least two weeks) wing/tail feathers of the *Psittaciformes* bird order (*Ara chloropterus*, *Cacatua galerita*, *Lorius garrulous*, *Amazona ochrocephala*, *Ara ararauna*, *Psittacus erithacus*, n=17) using the phenol-chloroform-isoamyl alcohol extraction method. The isolated DNAs was subjected to polymerase chain reaction with a primer commonly used for sex determination in birds. According to the nanodrop measurement results, it was revealed that sufficient DNA concentration to be used in DNA-based molecular studies can be obtained using the phenol-chloroform isolation method. Gender-specific band images were obtained from all DNA samples isolated according to PCR analysis performed for control purposes. As a result, it was revealed that DNA isolation can be done successfully with the Phenol-chloroform isolation method from the shed and waiting wing/tail feathers of the bird species *Psittaciformes*. It was concluded that non-invasively obtained DNAs can be used in molecular methods.

**Keywords:** *Bird, DNA isolation, feather, psittaciformes.*

## INTRODUCTION

The tissue sources for DNA used in molecular analysis in poultry are usually blood [1, 2, 3, 4], feather samples [2, 3, 4, 5, 6, 7, 8, 9], eggshell inner membrane [4, 7, 10] and slobber-saliva [11]. However, unlike in mammals, total blood is the most preferred tissue for DNA isolation in poultry because of the presence of nucleated erythrocytes [12]. In general, although it has been reported that no harm was given to the birds during blood collection [12, 13, 14], small amounts of blood can be taken from small passerine species and young ones. In addition, there is a risk of death if blood is not taken carefully [15]. For this reason, non-invasive methods that do not harm the bird should be preferred over invasive methods during the sampling phase for DNA isolation. Thus, DNA isolation from feathers has come to the fore. However, instead of removing fresh feathers by intervening with the bird, DNA isolation from spontaneously shed feathers without any

intervention to the animal is superior to other attempts to collect tissue samples in terms of both ease of application and animal welfare.

It is important to obtain the amount of DNA that will yield positive results at the molecular level for non-invasive gender determination. Each of the W chromosome-specific sequences used for gender determination in birds has been limited to one or a few taxonomically similar species. Therefore, ornithologists have searched for markers to differentiate bird species by gender [16, 17]. The W-linked chromosomal domain-helix DNA-binding (CHD-W) gene has been reported to be present on the W chromosome of all birds except ostriches and their relatives [18]. In-situ hybridization and linkage analysis data in chickens showed that this gene copy was located on the Z chromosome and was named as CHD1Z [19, 20]. The *CHD1W* and *CHD1Z* genes show high evolutionary conservation in both birds and mammals [21]. A molecular marker named 2550F/2718R was developed from the CHD1W and CHD1Z genes and can be successfully replicated in many bird species [21].

In this study, it was aimed to isolate high-quality DNA from shed and waiting feathers, which were taken from *Psittaciformes* bird order using the phenol-chloroform-isoamyl alcohol isolation method.

## MATERIALS AND METHODS

In this study, a total of 17 waited wings/tail feathers were obtained from *Psittaciformes* order kept in various commercial enterprises and zoo located in Ankara and Kayseri provinces as well as from the birds owned by people in these provinces. This study did not need to be confirmed by an ethical statement. Considering contamination risks, the collected feathers were placed in sterile bags and stored at room temperature. The waiting period of the feathers was at least two weeks (Table 1). General information about the samples is given in Table 1. At least one feather was collected from each bird, and the DNA isolation protocol was started with the one feather. The roots (calamus) of the collected wing and tail feathers were cut into small pieces using a scalpel and used for DNA isolation. DNA isolation was performed using the phenol-chloroform isoamyl alcohol method [22]. For each sample 2.5 units of Proteinase K enzyme was applied. The incubation temperature was 55 °C and the duration was 15 hours. The quality and concentration controls of the isolated DNAs were performed with a BioSpecnano (Shimadzu Ltd, Japan) spectrophotometer.

After the DNA isolation, PCR analysis was performed with a total volume of 20 µl reaction mixture that was prepared with $MgCl_2$ (2.0 mmol/L), 1 X buffer solution, dNTP (0.25 mmol/l), 0.5 U Taq DNA polymerase, 0.4 µl each forward 5′-GTTACTGATTCGTCTACGAGA-3′and reverse 5′-ATTGAAATGATCCAGTGCTTG-3′ primers (10 pmol) [19] and by adding 50 ng/µl DNA. The PCR reaction consisted of a total of 35 cycles of pre-denaturation at 95˚C for 5 minutes, at 95 ˚C for 30 seconds, at 55 ˚C for 30 seconds, at 72˚C for 30 seconds and finally terminated at 72 ˚C for 7 minutes. PCR reactions were performed in duplicate. The obtained PCR products were run on a 2% agarose gel at 120 V – 250 mA in the electrophoresis system and visualized under UV light. At the end of the electrophoresis process, the genders of the examined birds were determined by observing the presence of two bands of 450 bp (CHD1W) and 600 bp (CHD1Z) in females whereas a single band with a size of 600 bp in males.

***Table 1.*** *Information on the samples whose gender was determined by PCR method.*

| **Order** | **Sample number** | **Scientific Name** | **Total Size** | **Sample** | **PCR Results ♀** | **PCR Results ♂** | **State of knowing the gender** | **Mean ±St.D consantration of DNA form one shed feather (ng/µl)** | **Mean±St.D 260/280 OD** |
|---|---|---|---|---|---|---|---|---|---|
| *Psittaciformes* | | | | | | | | | |
| Red *Ara Macaw* | 1, 2 | *Ara chloropterus* | 2 | | 2 | | | 337.43±299.18 | 1.73±0.14 |
| Cockatoo | 3, 4, 5 | *Cacatua galerita* | 3 | | 1 | 2 | | 655.31±917.20 | 1.94±0.003 |
| Lory | *6, 7* | *Lorius garrulus* | 2 | | 2 | | | 718.25±961.01 | 2.05±0.007 |
| *Yellow-faced Amazon Parrot* | 8,9,10 | *Amazona ochrocephala* | 3 | | 2 | 1 | | 28.97±16.80 | 1.88±0.22 |
| Yellow-Blue Ara-Macaw | 11, 12, 13, 14<br>15 | *Ara ararauna* | 6 | | 3 | 3 | | 577.26±843.20 | 1.77±0.40 |
| Afrikan Gray parrot | 16, 17* | *Psittacus erithacus* | 2 | | 1 | 1 | 1 | 29.20±25.57 | 2.05±0.007 |

* *Gender was known*

After the DNA isolation, 260/280 OD values obtained as a result of spectrophotometric measurement and descriptive statistics of ng/µl concentration data were determined with the SPSS 22.0 statistical package program.

## RESULTS AND DISCUSSION

In the spectrophotometric measurements of DNAs obtained with the DNA isolation, the mean and standard deviation values of the quality (260/280 OD) and quantity of all samples were determined as 1.84 ± 0.05 and 422.59 ± 157.297 respectively.

The gender-related PCR bands of the samples after PCR analysis are given in Fig. 1. All samples yielded positive results for gender-related PCR bands.

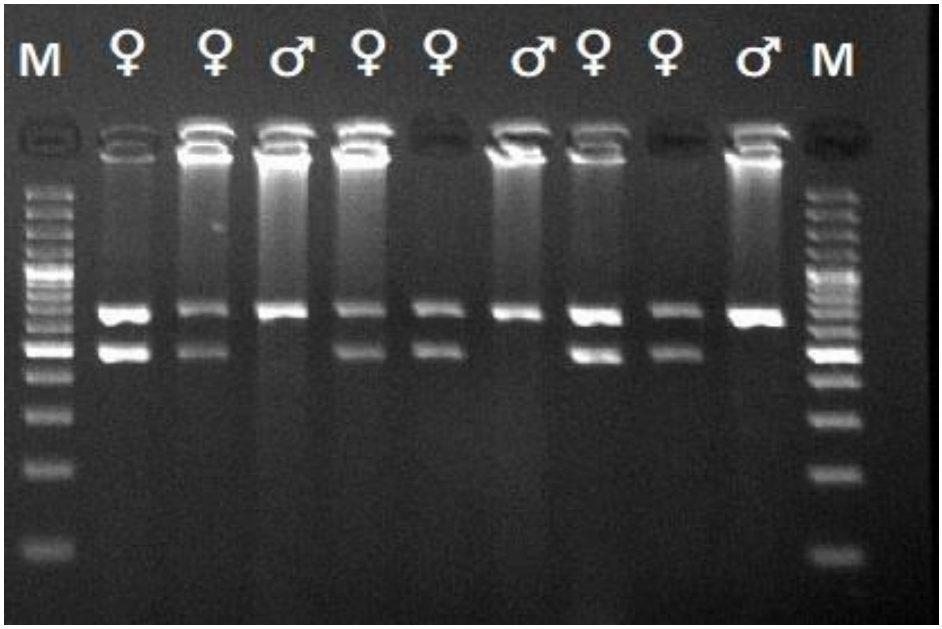


***Fig. 1.*** *PCR band images of feather samples. ♂: Male; ♀: Female; M: 100 bp DNA marker.*

There have been many studies reporting that feather samples can be used in DNA isolation for molecular genetic analyses in birds. It has been reported that the feather samples instead of blood samples in black-headed nightingales [23], feather samples of cockatiels (Nymphicus hollandicus) [6], and molted and long awaited feather samples of African gray parrots [18] can be used for gender determination. In a study of Vili et al. [24] investigating whether different physical conditions affect the DNA quality of feathers or not, it was reported that DNA isolation was successfully made from crow and goose feathers. In the literature review, it was observed that in the studies conducted in the last 10 years, the isolation was mostly carried out with a commercial kit, while the phenol-chloroform-isoamyl alcohol extraction method was used in some previous studies [6, 12, 19, 25]. Avanus and Koenhemsi [5] compared the success of DNA isolation using two different commercial kits in shed feathers of African gray parrot, and they reported that one of the kits gave positive results in shed and pending feathers whereas the other did not give positive results in feces contaminated and waited feathers. Therefore, they

suggested that fresh and clean feathers should be used in that kit for DNA isolation. However, as a result of DNA isolation made from shed feathers by phenol-chloroform extraction method in our study, DNA was obtained at a mean concentration of 422.59± 157.29 ng/µl from the examined samples that could be easily used in the PCR process.

Monge et al. [11] isolated DNA from the epithelial cells of the oral mucosa remaining on the fruits consumed by macaw parrots by using a commercial kit, and they obtained DNA at low quality and concentration. These authors [11] reported that the 260/280 OD absorbance and the concentration of DNA isolated with commercial kit from saliva swap samples taken from 15 fruit samples eaten by macaw parrots were 1.29±0.04 and 11.93±2.51 ng/µl respectively. However, in this study, in which DNA was isolated from one shed feather non-invasively by the phenol-chloroform method, 260/280 OD absorbance and concentration values were determined to be 1.73±0.14 and 337.43±299.18, respectively, in red Ara parrots, whereas 260/280 OD absorbance and concentration values were found to be 1.77±0.40 and 577.26±843.20, respectively, in yellow-blue Ara parrots. Therefore, since the method used in the present study was non-invasive, the amount and quality of DNA obtained from the shed feathers of macaw parrots was found to be sufficient for the analyses without the need for environmental DNA isolation from the saliva of the birds on the fruits they eat, as indicated in the aforementioned study by Monge et al. [11].

Many primers have been designed for both conventional PCR and RT-PCR for gender determination in birds using DNA. Among the primer pairs, the two primer pairs named P2/P3 and P2/P8 [18] and the primer pair named 1237L/1272H [26] as well as the primer pair named 2550F/2718R, which was designed by Fridolfsson and Ellegren [19] and was also used in this study are the most commonly used ones. The primer pair used in a gender determination study, which was carried out only with DNA obtained from the feathers of African gray parrots by Avanus and Koenhemsi [5], was also the primer pair, 2550F/2718R, used in this study that had been designed by Fridolfsson and Ellegren [19]. However, in the study of Avanus and Koenhemsi [5], isolation methods were performed with two different kits, and DNA quality and gender determination were determined only in African Gray parrots. However, in this study, sex determination in different *Psittaciformes* bird orders was performed with the same primer using the phenol-chloroform isolation method. Vucicevic et al. [27] was reported that the genders of *Amazona ochrocephala, Ara ararauna, Ara coloropterus gallerita, Psittacus Erithacus* from Psittaciformes family were successfully determined in protocols established with primer pairs 2550F/2718R [1999], which was also used in the present study.

## CONCLUSION

Because sex determination cannot be determined morphologically, especially for birds in the parrot order, molecular techniques are used to obtain DNA. In addition, DNA extraction is required in many different studies, including phylogenomic studies [28]. Non-invasive extraction of DNA for use in molecular reactions is important for animal welfare. The fact that the isolated DNA can be obtained after waiting for at least two weeks indicates that the intervention to the animal is minimized. In this study, since the sex of the parrots was not known, except for one sample, no special interpretation was made for sex determination, and only the results of the DNA isolated in molecular reactions were examined. In addition, studies specific to sex determination can be planned using different sex primers, including the primers studied. As a result of this study, it was

determined that DNA isolated from shed and wait wing/tail feather (at least two week) using phenol-cloroform method can be used in molecular reactions.

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**Conflict of Interest.** The authors declared that there is no conflict of interest.

**Authorship Contributions.** Concept: E.G.A, H.İ.K, M.T Design: M.T, H.İ.K., E.G.A Data Collection: M.T., H.İ.K Analysis: H.E., M.Y., Literature Search: H.İ.K., M.T., K.A., B.A. Writing: B.A, E.G.A., K.A.

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