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RESEARCH ARTICLE

Investigating The Usage of Molted Feather Samples as a DNA Source with Two Methods in Gender Identification of African Grey Parrot (*Psittacus erithacus*) by Molecular Analyses of CHDW and CHDZ Genes[#]

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ABSTRACT

The aim of this study was to evaluate the efficiency of two methods (method 1 and 2) for DNA isolation from molted feathers that were used in gender identification of African grey parrots (*Psittacus erithacus*). The molted feathers of twelve parrots were stored up to three months, two were contaminated with feces, further eight were stored up to five months; one was contaminated with feces totally feather samples of twenty parrots were used. Genomic DNA was isolated with method 1 successfully from the molted feathers of ten parrots that were stored for up to three months and free from feces. The differentiation of the gender that was made by amplification of chromo helicase-binding domain genes (CHDW and CHDZ), was resulted with two females and eight males. However no genomic DNAs were obtained from the feathers contaminated with feces or were stored up to five months with method 1 and none of the samples that were processed with method 2. Feces contamination and freshness of molted feathers affect the gender identification. In conclusion method 1 can be used in DNA isolation in order to perform gender identification from clean and fresh molted feathers in African grey parrots.

Keywords: Gender, African grey, parrot, molted feather, CHD.

Afrika Gri Papağanlarına (*Psittacus erithacus*) Ait Dökülmüş Tüy Örneklerinin İki Yöntem ile DNA Kaynağı Olarak CHDW ve CHDZ Genlerinin Moleküler Analizi ile Cinsiyet Tanımlamasında Kullanımının Araştırılması

ÖZ

Bu çalışmanın amacı Afrika gri papağanlarında (*Psittacus erithacus*) cinsiyet tayininde kullanmak üzere dökülmüş tüylerden DNA izolasyonunda iki metodun (metot 1 ve metot 2) etkinliğinin değerlendirilmesidir. Oniki tanesi üç aya kadar saklanmış; iki tanesi dışkıyla kontamine, ayrıca sekiz tanesi beş aya kadar saklanmış; biri dışkıyla kontamine olmak üzere toplam yirmi papağana ait dökülmüş tüy örnekleri kullanılmıştır. Üç aya kadar saklanmış ve dışkıdan ari on adet dökülmüş tüyden metot 1 ile genomik DNA başarı ile elde edilmiştir. Cinsiyet ayırımı kromo helikaz-bağlanma bölgesi genlerinin (CHDW ve CHDZ) çoğaltılması ile iki dişi sekiz erkek olarak sonuçlanmıştır. Ancak genomik DNA metot 1 ile dışkı ile kontamine olmuş tüyler ile birlikte beş aya kadar saklanmış olan tüylerin hiçbirinden, ayrıca metot 2 ile tüy örneklerinin tamamından elde edilememiştir. Dökülmüş tüylerin dışkı ile kontaminasyonu ve tazeliği cinsiyet tayininin başarısını etkilemektedir. Sonuç olarak metot 1, Afrika gri papağanlarında temiz ve taze dökülmüş tüylerden DNA izolasyonu yapılarak cinsiyet tayininde kullanılabilir.

Anahtar Kelimeler: Cinsiyet, Afrika grisi, papağan, dökülmüş tüy, CHD

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INTRODUCTION

African grey (*Psittacus erithacus*) is one of the most popular companion parrots in Turkey. Determining the gender of avian species is generally difficult before puberty. However in monomorphic avian species it is even difficult after puberty (Cerit and Avanus 2007[a]). Owls, ducks, nestling turkeys, geese, and parrots are some of the monomorphic avian species that their gender identification is challenging due to their monomorphological features. This also affects wildlife conservation and breeding studies of parrots performed by scientists, breeders and owners (Griffiths et al. 1995, Griffiths et al. 1998). If breeders hesitate about the gender of the candidate parents in order to perform nestlings, their success and required time duration in obtaining newborns will be affected (Cerit and Avanus 2007[b]). Avian gender chromosomes Z and W are differently evolved in birds compare to mammalian gender chromosomes X and Y (Ellegren and Carmichael 2001). As opposed to in mammals, in avian species heterogametic (ZW) one is the female and homogametic (ZZ) one is the male (Handley et al. 2004). The most critical question is how two types of gender chromosomes play a role in gender identification of birds. Although W chromosome is female specific, its structure resembles to mammalian male Y chromosome. Chromo helicase DNA binding protein (CHDW) and ATP synthesis α-sub unit (ATP5A1W) genes were defined on the W chromosome. The non-recombined part of the W chromosome includes these both genes and Z chromosome contains their similar homologues (CHDZ and ATP5A1Z). Intron region size varies between CHDW and CHDZ genes. Therefore it also differs between male (ZZ) and female (ZW) birds. Differentiation of genders with molecular analyses are based on this variation (Griffiths and Tiwari 1993, Griffiths and Korn 1997, Fridolfsson and Ellegren 2000, Ellegren 2001). The aim of this study was to evaluate the usability of molted feather samples as a DNA source by two methods to perform gender identification in African grey parrot (*Psittacus erithacus*) by genetic analyses of CHDW and CHDZ genes.

MATERIAL AND METHODS

This study was conducted on molted feather samples of twenty African grey parrots. Molted feathers were collected from individual cages without giving any harm to parrots.

Feather Samples

For each parrot, feather samples were taken from individual cages into separate clean plastic bags and stored in the dark at room temperature. The feather samples of twelve parrots were stored up to three months, two were contaminated with feces. Moreover feather samples of eight parrots were stored up for five months, one of them was contaminated with feces.

DNA Isolation From Feather Samples

The calamuses of the feathers were used as a DNA source (Figure 1). According to the size of the calamuses, 3-5 feather samples were used from each parrot. The feathers were cut with sterile scalpel tips. The calamuses were removed, chocked and put in to labeled 1.5 ml sterile centrifuge tubes in order to perform DNA isolation. Two methods used for DNA isolation from feather samples were named as method 1 and method 2.

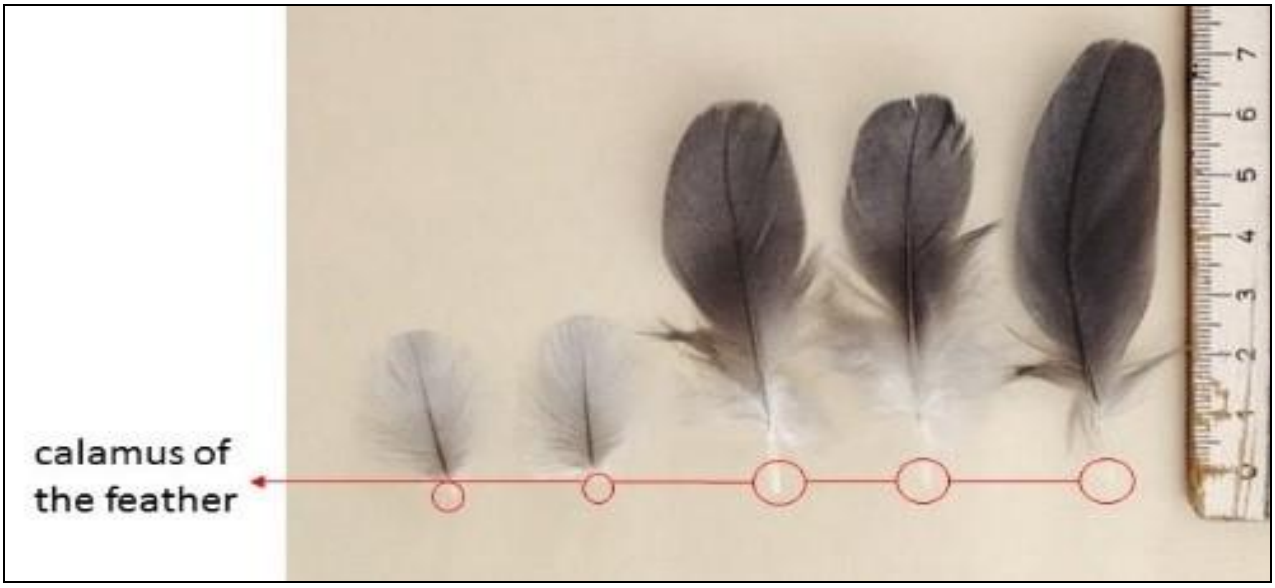

Figure 1: The calamus of molted feather samples in an African grey parrot that were used as a DNA source.

Method 1

The first DNA isolation method used for feather samples of twenty African grey parrots was a modified protocol of DNeasy® Blood and Tissue kit (Qiagen, Valencia, California, USA) (Bush et al. 2005). Kit suggests incubation duration for lysis until animal tissue dissapers, therefore an overnight incubation was performed at 55°C in incubator in this study. Kit contains a ready to use 20 mg/ml proteinase K solution, but it was not enough for lysis procedure, therefore 25 mg/ml proteinase K was prepared. All other steps that described for animal tissues in the user manual of the kit were followed (http://diagnostics1.com/MANUAL/General_Qiagen.pdf).

Method 2

The second DNA isolation method from the feather samples of twenty African grey parrots was a modified protocol of PureLink® Genomic DNA Kits (Invitrogen, California, USA). This kit contains a ready to use 20 mg/ml proteinase K solution. However lysis step was not sucessfuly completed with 20 mg/ml, therefore two proteinase K solutions were prepared in different concentrations (25 mg/ml and 30 mg/ml). Incubation was performed overnight at 55°C. All other steps for animal tissues were followed as described in the kit's user manual (https://tools.thermofisher.com/content/sfs/manuals/purelink_genomic_man.pdf).

Visualizing of genomic DNA

After performing DNA isolation with method 1 and method 2, all samples were load in to 0.8% agarose gel electrophoresis and genomic DNAs were visualized on UV transilluminator.

Amplification of CHD genes

The region of the CHD genes were amplified with 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') primer pairs (Fridolfsson and Ellegren 1999). The PCR amplification was performed in a total volume of 25 µl consists from 5 µl Taq PCR Master Mix (200 U/ml Ultra-Pure Taq DNA Polymerase, 1.25 mM dNTPs, 10 mM $MgCl_2$; Geneaid Biotech™, Taiwan), 0.5 µl 20 pmol of each primer, 3 µl genomic DNA (100 ng) and 16 µl dH_2O (AccuGENE™, Lonza, Belgium). Amplification was performed with the following conditions; denaturing at 95°C in 4 min, 35 cycles of 95°C in 30 sec, 55°C in 30 sec, 72°C in 45 sec and final extension at 72°C in 5 min in thermal cycler machine (Bio-Rad T100, Bio-Rad Laboratories Inc., CA, USA). The PCR products were loaded into 3% agarose gel electrophoresis and they were visualized on UV transilluminator.

RESULTS

Method 1

Genomic DNAs were successfully isolated from feather samples of ten parrots that were stored up to three months and they were observed on 0.8% agarose gel electrophoresis. After performing the amplification of CHD genes, single (600bp) and double band (600bp and 450bp) patterns were observed on 3% agarose gel electrophoresis for male and female birds respectively. The gender of ten African grey parrot were identified as eight male and two female (Figure 2). Genomic DNA isolation with method 1 could not successfully performed not only for the feather samples of two parrots that were contaminated with feces, but also eight parrots that their feather samples were stored up to five months.

Figure 2. Gender identification was performed on 3% agarose gel electrophoresis. Out of ten parrots two were identified as female and eight as male from molted feather samples with using method 1. Female and male African grey parrots were observed with double (450bp and 600bp) and single (600bp) band patterns respectively (L: 100bp DNA ladder).

Method 2

After performing DNA isolation from feather samples of twenty African grey parrots with method 2, no genomic DNA bands were observed on 0.8% agarose gel. Even so PCR amplification was applied to the samples and no amplicons were observed on 3% agarose gel electrophoresis as expected.

DISCUSSION

The balance of the female/male ratio is important in a small population of companion parrots. If gender identification of parrots are not clear reproduction process could be longer than predicted which will also cause significant financial losses (Cerit and Avanus 2007[b]). However gender identification by the inspection of morphological characteristics is challenging in African grey parrots since they are one of the monomorphic avian species. Before the DNA analysis method was developed, gender of monomorphic birds was determined by laparoscopic or karyotyping methods. But birds could have been harmed or even been killed during the implementation of these methods (Trewick 1996, Heinsohn et al. 1997, Saino et al. 1999).

After developments in molecular genetics methods, gender identification in monomorphic birds was begun to analyze with DNA typing of CHDW and CHDZ gender chromosomes (Ellegren 1996). Isolation of genomic DNA for gender identification in parrots by molecular analyses of CHD genes can be performed by using various materials such as blood samples, plucked or molted feathers. Blood and plucked feathers were the most preferred materials in avian DNA isolation (Harvey et al. 2006, Bosnjak et al. 2013, Vucicevic et al. 2013, Bogovic et al. 2017). Since FTA cards could lead to a high risk of cross contamination, EDTA tubes are advisable for collecting blood samples (Suriyaphol et al. 2014). However, both collecting blood samples and plucking feathers are stressful for parrots (Harvey et al. 2006), the molted feathers could be an alternative, less stressful and painless material for obtaining genomic DNA in African grey parrots. Buccal swabs and feces can also be suggested as alternative DNA sources. Whereas collecting buccal swabs is also stressful for the bird and using feces samples does not result with enough DNA yield to perform gender identification in avian species (Bogovic et al. 2016). Consequently taking molted feather samples is easier for the researcher and more comfortable for the parrot compared to collecting blood samples, plucking feathers and taking buccal swabs. The feather samples in this study were kept, until all twenty feather samples were collected which took approximately five months. Molted feathers of six parrots were collected within a month and analyzed directly and all were resulted by successful DNA isolation with method 1. The other four successful isolations were performed with the molted feathers that were stored up for three months. But DNA from the feathers older than three months was not isolated qualified enough to perform PCR for gender identification. For a good yield of genomic DNA, feather samples are need to be fresh and this affects the isolation and quality of genomic DNA.

Feather samples of three parrots were contaminated with feces, two of them were stored for three months and the other for five months. Genomic DNA was not obtained from none of those molted feathers. Isolation of DNA could have been much more successful if all the feather samples were free from feces. If molted feather is planning to be used for gender identification, one of the key factors is the housing the parrot in an individual cage. Therefore samples were not collected from pair housing cages. Otherwise feather samples might be mislabeled and gender identification can be misidentified. Amplification of CHDW and CHDZ genes in order to gender identification was performed successfully with 2550F and 2718R primer pairs. The sizes of the PCR products of this study for CHD Z (600bp) and CHDW (450bp) genes were conform to previous study performed by Fridolffson and Ellegren (1999) in different bird species since CHD gene is a conserved region between avian and mammalian species (Stevanov-Pavlovic 2013).

In conclusion, the primer pairs used in this study (2550F and 2718R) are usable in gender identification of African grey parrot with DNA analyzes of CHDW and CHDZ genes. If molted feathers were picked as a material for gender identification, method 1 can suggested to be used for DNA isolation but feather samples should be fresh and clean. Further practical, efficient and painless DNA isolation methods should be developed for gender identification in monomorphic bird species from molted feather and feces. However these samples should be obtained fresh, otherwise performing a successful DNA isolation could be challenging.

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