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Some Parts of the Feather Can be a Non-Invasive Genetic Sample for Sexing in Avian?

Research Article

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ABSTRACT

The current animal research investigation emphasizes animal wellbeing. Therefore, the present study recommends using non-invasive sampling procedures in scientific studies. The purpose of this study was to evaluate DNA extracted from 7 different parts of the feather (calamus tip, rachis (I-III) and barbs (I-III)) for sex identification using polymerase chain reaction (PCR) amplification. The study results showed that the calamus tip had the highest DNA concentration. DNA extracted from rachis and barbs can also be amplified and use for sex determination. Extracted DNA from the calamus tip, rachis (I), and barbs (I) showed accurate results for amplified PCR products when compared to the sex of known samples. The findings revealed that, in addition to the calamus tip, the rachis and barbs on the lower part of the feather surrounding on the calamus can be used to determine sex. Calamus tip collection by plucking is an invasive technique that could result in contusion and infection. The non-invasive sample collection method of cutting a portion of the feather is one option for supporting animal welfare guidelines. Furthermore, the non-invasive method presented in the study can be used to collect samples in other branches of molecular biology.

KEY WORDS bird, feather, PCR, sex identification.

INTRODUCTION

Sex determination in birds is critical for biological studies such as behavior and ecology, as well as commercial bird mating. Identification of sex from external features is often mistaken because the pet birds are monomorphic in appearance (Witte and Curio, 1999). In other words, the male and female birds are similar in external appearance. However, genders can be distinguished in dimorphic birds such as peacocks and ducks. There are currently several methods used for determining a bird's sex such as examination of the pelvic girdle (Anten-Houston *et al*. 2017), cloaca (Boersma and Davies, 1987), laparoscopy (Richner, 1989) and blood venepuncture for determined sex chromosome (Ortega *et al*. 2017). All of the techniques mentioned above are hazardous to the animal. It's easy to startle birds. As a result, plucking feathers can make the bird anxious and stressed (Khumput *et al*. 2019), reducing its market value (Lumeij and Hommers, 2008). Polymerase chain reaction (PCR) techniques are currently used in combination with gel electrophoresis. It is a widely used method for sex identification of birds, based on differentiation of the intron region in chromo-helicase-DNA binding protein (CHD) genes which located on the bird sex chromosomes, both Z and W chromosomes. Female birds have a ZW chromosome system, which leads to the CHD-Z and CHD-W genes, while male birds have a ZZ chromosome system, therefore male birds only have CHD-Z genes (Griffiths and Korn, 1997). Many studies have found that using a separation amplified product of Z and W chromosomes, it is possible to sex non-ratite birds. The universal primer pair (2550F/2718R) have been used for PCR amplification to

identify sex of birds, in the orders of Pelecaniformes (Kocijan *et al*. 2011), Ciconiiformes (Marija *et al*. 2013), Accipitriformes (Çakmak *et al*. 2017) and Psittaciformes (Miyaki *et al*. 1998) However, one of the concerns for animal welfare is the sampling techniques for DNA extraction. The conventional DNA extraction required blood (Quintana *et al*. 2008) and calamus (Changtor and Yimtragool, 2020; Harvey *et al*. 2006; Avanus and Koenhemsi, 2018; Purwaningrum *et al*. 2019), because the DNA extracted from blood and calamus is highly pure and sufficient for use. However, blood sampling and plucking are dangerous techniques for animals. Prolonged periods of handling animals for the purpose of pecking feathers can cause stress. It can also lead to injury or infection in birds. Bird identification and sexing were attempted using non-invasive specimens, such as eggshell waste (Trimbos *et al*. 2009), oral mucosa (Wellbrock *et al*. 2012), urine (Nota and Takenaka, 1999) and faeces (Segelbacher and Steinbrück, 2001) . There were problems with the amount of DNA yield and protein contamination that interfere and making gender determination impossible. Some part of feathers obtained without invasive sampling is another good example for retrieving DNA for molecular genetics assays (Speller *et al*. 2011). Previously, sex identification of certain bird species used only feather barb for DNA amplification using PCR. (Boonseub *et al*. 2012). Feather development begins in the dermis and is nourished by blood vessels. Feather development also leads to the formation of the rachis and barb. The growth and development of the rachis and feather barbs continue throughout the life of the feather. The preceding part of the rachis and feather barb will be at the top of the feather as it develops in length. This suggests that feather barbs can be used as non-invasive samples for bird sex identification, although various parts of the feather may have varied possibilities for DNA extraction and amplification. Therefore, the objective of this study is to investigate the feasibility of using various parts of feather for bird sex determining by PCR technique without causing harm to the animal.

MATERIALS AND METHODS

Sample collection and determination of feather part for DNA extraction

In this study, contour feathers were collected form 10 green-cheeked parakeets (*Pyrrhura molinae*). Parakeets were provided by the sex identification of pet birds project, Department of Biology, Faculty of Science, Naresuan University. The four centimeters in length feathers were plucked from the bottom of the bird and divided into 7 parts of the feathers: calamus tip, rachis (I-III) and barbs (I-III))Figure 1). Protocols for the animal experiments were approved by Naresuan University Animal Ethics proved by Naresuan University Animal Ethics Committee with registration number NU-AEE630803.

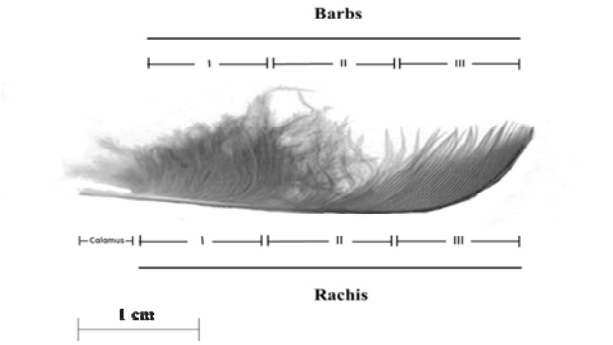

Figure 1 The parts of feather for DNA extraction

DNA extraction and examination of DNA quality

Each part of feather was lysed in a 1.5 mL microcentrifuge tube. To lysate, 300 µL of the lysis buffer (1×TNE (100 mM NaCl, 50 mM Tris and 25 mM EDTA), 30 µL of 1 M Tris-HCl, 22.5 µL of 25 mg/mL Proteinase K, 5 µL of 25% SDS and newly prepared 40 µL of 1 M DTT) were added and incubated at 55 ˚C in a hot air oven for 7 days and vortexed once daily as described by Bayard de Volo *et al*. (2008). After that, protein from the solution was precipitated using 300 µL of 7.5 M ammonium acetate and incubated at 4 ˚C for 30 min. The debris was removed by centrifugation at 13000 rpm for 5 min at 4 ˚C and 400 µL of supernatant was transferred into a new tube containing 300 µL of isopropanol. The solutions were mixed and incubated together at -20 ˚C overnight. The tubes were centrifuged at 13000 rpm for 30 min at 4 ˚C, and supernatant was discarded. The pellet was washed using 70% ethanol and centrifuged at 13000 rpm for 2 min at 4 ˚C. The pellet was air-dried at room temperature for 5 min and resuspended in 10 µL of nuclease-free water. DNA concentration and purity were measured using a NanoDrop 2000 (Thermo Scientific™) at wavelengths of 260 and 280 nm.

DNA amplification and analysis with gel electrophoresis

Extracted DNA was amplified using MyTaq^{TM} HS Mix (Hot start PCR, Bioline) containing 5 µL of 2 × buffer, 10 pmol of each primer 2550F (5'-GTTACTGATTCGTCTACGAGA-3')/2718R (5'-ATTGAAATGAT CCAGTGCTTG-3') (Fridolfsson and Ellegren, 1999) and 1 µL of template DNA. Nuclease-free water was added to the solution to achieve a total volume of 10 uL. The PCR amplification was carried out by 35 cycles, of which denaturation at 95 ˚C for 45 sec, annealing at 58 ˚C for 45 sec and extension at 72 ˚C for 45 sec with an initial denaturation at 95 ˚C for 5 min before cycling and final extension at 72 ˚C for 5 min after cycling with a T100

thermal cycler (BIO-RAD). The amplified PCR products were separated by 2% agarose gel in 1 × TAE buffer and electrophoresis at 120 V for 35 min. The PCR amplicons were visualized under UV light with 1 µg/mL ethidium bromide staining. The size of the PCR amplicons was determined by comparing them to the OneMARK 100 DNA Ladder RTU (GeneDirex). All gel electrophoresis photographs were taken with a camera

Statistical analysis

The DNA concentration and purity were compared with the Kruskal-Wallis One-Way Analysis of Variance and the pairwise was tested using the Mann–Whitney U test at 95% confidence with Past3 version 3.22. The graph was created using RStudio 4.0.5. The accuracy of gender identification was calculated by number of bird's sex identify using PCR technique dividing by total number of samples used for examination.

RESULTS AND DISCUSSION

As described in the method section, DNA was extracted from various parts of ten contour feathers using lysis buffer containing SDS, DTT, and proteinase K. The variation in DNA concentration and purity were tested using Kruskal-Wallis, and the results revealed a statistically significant difference among groups. The different between each group was compared with Mann–Whitney U test, as shown in Figure 2. The result showed that DNA could be extracted from all parts of the feather. The highest concentration of DNA was obtained from feather's calamus, which was approximately 57.46 ± 5.27 ng/µL. Therewith, the extracted DNA concentrations in the rachis (I), rachis (II), and feather barbs (I) were 25.54 ± 6.27, 19.02 ± 1.56, and 19.33 ± 2.48 ng/µL, respectively. The quality of DNA, as measured by purity values (OD260/280), did not differ statistically between feather parts. (mean range: 1.17-1.43) (Figure 2).

The yield and quality of extracted DNA from the feather depends on the DNA extraction method. The use of lysis buffer in DNA extraction methods containing high concentrations of DTT, Proteinase K, and SDS has shown increase in DNA yield and purity (Bayard De Volo *et al*. 2008; Kilatsih *et al*. 2020). Begovic *et al*. (2017) and Peters *et al*. (2020) compared the efficiency of this method to a commercial kit. The concentration of extracted DNA using lysis buffer method is equal to the commercial kit. DTT was used to break down the disulfide bond in the protein structure. Meanwhile, Proteinase K was used to disintegrate protein in feathers and SDS enabled the digestion of protein by changing total electric charge in feathers (Campos and Gilbert, 2011).

Feathers are the excellent source of DNA because they contain the cell such as epithelial and blood cells. Furthermore, the majority of avian erythrocytes are nucleated and retain the nucleus throughout the circulatory lifespan of the cell (Clark, 2015). As a result, blood cells in feathers can be utilized to extract higher amount of DNA. It has been reported that DNA extracted from fresh feathers has higher quality DNA than dry feathers (Bello *et al*. 2001; Andleeb *et al*. 2012). The OD260/280 ratio for pure DNA is typically considered to be between 1.8 and 2.0. However, extracted nucleic acid samples are frequently contaminated with other molecules (i.e. proteins, organic compounds, others). When the OD260/280 is less than 1.8, it indicates that protein contaminants have incorporated in the DNA sample. The medulla, cuticle, cortex, and pigment granules are the four structures that make up the keratin and melanin granules (Wilson *et al*. 1995). The results of purity values in this study were not statistically different. This suggests that the ability of the DNA extraction method could eliminate the protein component which is essential for DNA amplification.

The results of DNA extraction from different parts of the feather and amplification with the PCR technique for sex identification revealed that the calamus tip, rachis (I), and feather barb (I) could be used as a source of DNA. The calamus, the rachis (I) and the barb (II) were 100 percent accurate in determining the sexes. The rachis (II) was found to be capable of extracting DNA to identify sexes, despite the fact that only one sample accounted for 20 percent (Table 1). Our findings demonstrated that DNA extracted from the calamus, rachis (I), rachis (II), and feather barbs (I) can be used for DNA amplification. These findings are also consistent with previous studies in which researchers used feather as a potential source for DNA extraction (Bayard de Volo *et al*. 2008; Andleeb *et al*. 2012; Avanus and Koenhemsi, 2018; Purwaningrum *et al*. 2019). Especially the study of DNA extraction used only some parts of the feather such as calamus (Presti *et al*. 2013; Avanus and Koenhemsi, 2018; Changtor and Yimtragool, 2020) or feather barbs (Speller *et al*. 2011). The findings suggest that a part of the feather could be used as a source of DNA for biomolecular research. Only a few barbs can be used for DNA amplification and are suitable for DNA sequencing, as highlighted by Speller *et al*. (2011) and also confidently presented in the current study. These findings lend credence to the idea that feather barbs can be used as DNA sources. Upon reviewing previous research, no data on the benefit of rachis for DNA extraction was found. However, rachis and feather barbs develop from the same tissue. In addition, the development of feather barbs occurs after the rachis matures.

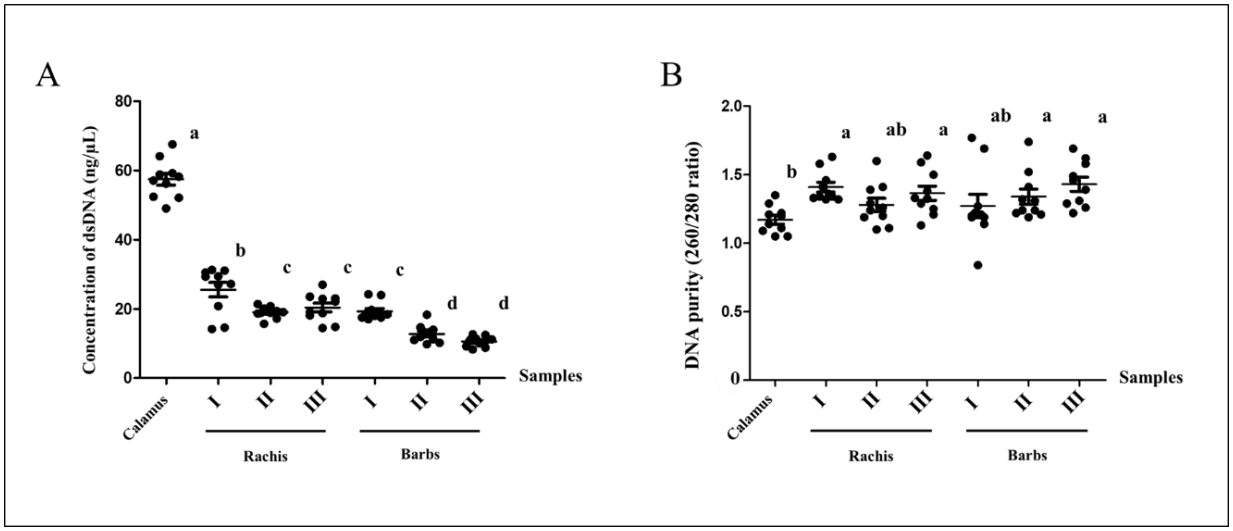

Figure 2 Concentration (A) and purity (B) of extracted DNA from the parts of feather (N=10) by using lysis buffer, and the different letter show statistically significant difference among group test with Mann–Whitney U
The dot (•) is individual sample which represents each part of feathers

Table 1 The percentage of successful sex identify by PCR

Part of feather	Sample	Sex identification by PCR		Percentage
		Male	Female	
Calamus tip		4/4	6/6	100
Rachis	I	4/4	6/6	100
	II	0/4	2/6	20
	III	0/4	0/6	0
Barbs	I	4/4	6/6	100
	II	0/4	0/6	0
	III	0/4	0/6	0

The blood vessels that nourish the rachis and feather barbs gradually disappears. After that, a new section of the feather develops at the bottom that contains blood vessels. The calamus is the area adjacent to the bird's skin cells, which is nourished by a large number of blood vessels. As a result, the rachis has the potential to be a DNA source which can be used for PCR amplification, similar to a previous study that extracted DNA from feather barbs and used it in PCR techniques. The rachis section is closer to the calamus. As a result, DNA extracted from the rachis section should contain more DNA than feather barbs. Finally, the failure of rachis and barbs (II-III) DNA amplification may be due to the location of this sections being away from the dermal papilla layer, resulting in low DNA concentration, as also evidenced by the current study. In conclusion, rachis has the potential to be DNA sources, and the benefit of rachis is that it can avoid contamination of foreign DNA because the rachis still inside of feather, allowing less contact with the environment. In this study, the PCR products of the female green-cheeked parakeet (*P. amolinae*) were found to have two bands of approximately 450 bp and 650 bp. Only one band of approximately 650 bp wasfound in males (Figure 3, Table 1).

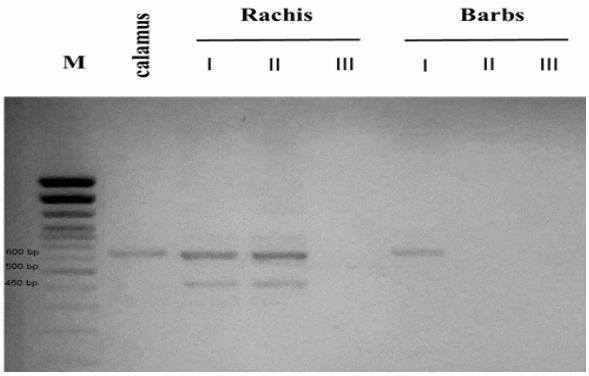

Figure 3 Agarose gel electrophoresis with ethidium bromide staining shows sex determination of Green-cheeked parakeet (*Pyrrhura molinae*) by PCR
Lane 1–DNA ladder, lane 2-calamus tip, lane 3-5–rachis (I-III) and lane 6-8 – barbs (I-III)

The amplicon fragments found in this study are similar in size to those found in other birds in this genus (Miyaki *et al*. 1998). The 2550F/2718R primer pair was used to demonstrate that the feather part can be successfully used for DNA amplification. The PCR technique is popular for bird sex identification because it is simple to perform, has high accuracy, and sample collection for DNA amplification using PCR can reduce bird injury. The PCR technique requires DNA extracted from any bird tissues including blood, feather (Jensen *et al*. 2003; Harvey *et al*. 2006), wing tissue from dead birds (Morinha *et al*. 2011), egg shells (Bush *et al*. 2005) and fecal matter (Idaghdour *et al*. 2003).

The most common sex identification samples are blood and calamus from plucked feathers (Changtor and Yimtragool, 2020; Presti *et al*. 2013). Although these methods provide a sufficient amount of DNA and are suitable for biomolecular work, they are invasive and may cause harm to the bird. Some feather sections can be used in place of these samples for DNA extraction. Therefore, it is suitable for biomolecular studies that require a high DNA concentration, such as investigating the genetic diversity of birds. The rachis and feather barbs are feather parts that can be collected without invasive the bird. Furthermore, the benefit of this research can explain that the unnecessary part of the feather with high protein and low DNA can be avoided to improve the quality of extracted DNA and makes it easier to use in molecular work. However, extracting DNA from feather parts necessitates a method that maximizes DNA yield while minimizing protein contamination, which can lead to false positives.

CONCLUSION

This study demonstrated that the parts of the rachis and barbs located on lower part of the feather bordering on the calamus can be used as a non-invasive genetic sample to replace blood sampling or feather plucking for sex determination in bird to reduce harmful operation. Our discovery not only advances the study of bird sex identification, but it can also be applied to field studies or other molecular work in avian, particularly for endangered species.

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