ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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OPEN ACCESS International Journal of Poultry Science

ISSN 1682-8356
DOI: 10.3923/ijps.2020.244.251

Research Article
Comparison of DNA Extraction Methods and Selection of Primer Sets for Sex Identification of the Red-Whiskered Bulbul (*Pycnonotus jocosus*)

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Abstract

Background and Objective: The red-whiskered bulbul (*Pycnonotus jocosus*) is an avian species that is considered to have economic value. Problem of propagation are often encountered by breeders as birds of the same gender placed together do not mate and reproduce. Polymerase chain reaction (PCR), as a popular biomolecular technique was used to solve this problem. This study aimed to compare DNA extraction methods and select the primer sets for sex identification using PCR. **Materials and Methods:** A total of 5 (blood/feather calamus) samples were subjected to DNA extraction with different methods: blood using lysis buffer and feather calamus using lysis buffer and alkaline lysis. DNA fragments were performed by PCR using 4 primer sets CHD1F/CHD1R, 2550F/2718R, P2/P8 and P2/NP/MP. The PCR products of each primer set were sequenced to determine a map location of 4 primer sets on the chromo-helicase DNA-binding (CHD) gene. **Results:** The feather calamus with lysis buffer method rendered the highest DNA concentration. No statistically significant differences were found in the purity of DNA obtained from the three methods of extraction. Extracted DNAs were used in sex identification at 100% usability. Results showed the CHD1F/CHD1R and P2/P8 primer sets accurately identified the sex of the red-whiskered bulbul, while the CHD1F/CHD1R more clearly separated PCR products by agarose gel electrophoresis compared with the other three primers. **Conclusion:** CHD1F/CHD1R was identified as a suitable primer for sex identification in this species and may also be applied to related species.

Key words: Red-whiskered bulbul, sex identification, blood, feather calamus, DNA extraction, primer selection, PCR

Received: February 25, 2020 Accepted: April 26, 2020 Published: May 15, 2020

Citation: Phanupong Changtor and Nonglak Yimtragool, 2020. Comparison of DNA extraction methods and selection of primer sets for sex identification of the red-whiskered bulbul (*Pycnonotus jocosus*). Int. J. Poult. Sci., 19: 244-251.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Red-whiskered bulbuls (*Pycnonotus jocosus*) are popular pets in Thailand because the birds emit melodious vocals. Singing contests of this bird species have established the red-whiskered bulbul as a high-value bird, fetching 500-5,000 baht in the open market with large demand for young birds throughout the country[1]. In 2013, the red-whiskered bulbul was registered as a legally protected species but commercial breeding is still allowed. Red-whiskered bulbuls mate by internal fertilization. In the wild, red-whiskered bulbuls mate and breed naturally but when raised in captivity, mating opportunities may be limited. Sometimes breeders have two specimens of the same sex in one cage, thus mating and breeding are not possible. Some breeders lack the necessary expertise to identify the sex of specimens by their external appearance to make breeding a success. Several methods are used for sex identification in poultry. These include examination of the pelvic girdle[2] and the cloaca[3]. These methods can help in sexing the birds but may be harmful because of the need for an extensive tactile examination. Birds are easily frightened and handling may cause increased anxiety. They may even engage in self-injuring activities. Another way of identifying the sex of the birds is to observe their behavior; however, this method may not be entirely accurate[4].

Nowadays, the chromo-helicase DNA-binding (CHD) gene is used for bird sex identification[5]. Various molecular techniques have been applied in the sex determination of birds using CHD gene differences on their sex chromosomes. The sex determination system of birds uses the ZZ and ZW chromosomes. The CHD genes on the Z and W chromosomes differ in parts that are not decoded and this may be used to identify bird gender. Male birds only have the CHD gene on the Z chromosome (CHD-Z), while female birds have the CHD gene on both the Z and W chromosomes (CHD-Z and CHD-W). Various molecular techniques have been used to expand gene quantity and classify both genes such as SSCP[6], RFLP[7], RAPD[8] and AFLP[9]. However, basic biomolecular techniques such as PCR remain popular in bird sex identification[10]. The primer is normally designed to produce two DNA fragment characteristics as (1) The product of the CHD-W gene is smaller than that of the CHD-Z gene, such as the primer set 2550F/2718R[11] and (2) The product of the CHD-W gene is larger than that of the CHD-Z gene, such as primer P2/P8[12]. Each primer is suitable for some bird species. The 2550F/2718R primer can separate the sex of birds in the orders Pelecaniformes and Ciconiiformes with 100% accuracy but in Passeriformes, the order of the red-whiskered bulbul, only 3.6% of the genders can be correctly identified[13]. Sex separation of the red-whiskered bulbul using primer P2/P8 determined positive results[14]; however, this primer predisposes to error because the difference in output only has a length of 30-50 bp. The output isolation method by agarose electrophoresis often results in fragments that appear to be the same and this can lead to incorrect sex identification[15].

Normally, sex determination of birds by molecular biology techniques is performed by collecting blood samples and feather calamus. DNA extraction from the blood is easy; however, the feather calamus contain high amounts of keratin proteins and pigments and extracting DNA requires protein precipitation and DNA purification which are complicated procedures. Yield and quality of DNA required for PCR and sex identification is often unsuccessful. Several methods have been proposed to extract DNA from tissues that contain high protein. Therefore, the aims of this study were to compare quality and quantity of DNA extraction from blood and feather calamus using different extraction methods and select a suitable primer set for sex identification of the red-whiskered bulbul by PCR. The results of this study can be used in the bird breeding industry and also as a means to identify DNA from different sources.

MATERIALS AND METHODS

Sample collection: Blood samples and plucked feathers were collected from 5 red-whiskered bulbuls (*P. jocosus*, N = 5). The sex of the subject birds was already known before the tests were undertaken. Blood samples were collected by toenail clipping and sample volumes of 5-10 µL were absorbed using paper with hemostasis performed following the method of Sakas[16]. The blood samples were dried at room temperature. Plucked feather samples were collected from the vent area under the tail cover and placed in a plastic bag. All samples were stored at -20°C until required for use. All protocols for the animal experiments were approved by Naresuan University Animal Ethics Committee, registration number NU-AE620512.

DNA extraction

Blood sample with lysis buffer method (I): The paper with 5-10 µL of blood sample was transferred to a 1.5 mL microcentrifuge tube into which a 300 µL aliquot of lysis buffer had been added. The lysis buffer included 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^{-1} Proteinase k, 5 µL of 25% SDS and newly prepared 40 µL of 1 M DTT following Bayard de Volo *et al.*[17]. The paper was removed after

incubation at 55°C for 10 min and the solution was further incubated at 55°C for 2 h. The protein was then precipitated with 300 µL of 7.5 M ammonium acetate and further incubated at 4°C for 30 min, followed by centrifuging for 5 min at 13,000 rpm. The supernatant was transferred to a new tube containing 300 µL of isopropanol and incubated in a freezer overnight. The solution was then centrifuged at 4°C 13,000-16,000 rpm for 30 min. The supernatant was poured off, 400 µL of 70% EtOH was added and the mixture was centrifuged for 2 min. The DNA was air-dried to remove excess EtOH and DNA pellets were rehydrated with 20 µL of nuclease-free water and stored at -20°C until required for analysis.

Feather calamus with lysis buffer (II): A half inch section was cut from a feather calamus, placed in a 1.5 mL microcentrifuge tube containing lysis buffer as detailed above (I) and incubated at 55°C for 5-7 days. After the protein and DNA precipitation, the DNA pellet was rehydrated with 10 µL of nuclease-free water and stored at -20 ° C until required for analysis.

Feather calamus with alkaline lysis (III): A half inch section was cut from a feather calamus, placed in a 1.5 mL microcentrifuge tube containing 75 µL of alkaline lysis (25 mM NaOH and 0.2 mM disodium EDTA) and incubated at 95°C for 1 h. After heating, the sample was cooled and neutralized with 75 µL of 40 mM Tris-HCl. The solution was kept at 4°C until required for analysis according to the method as described by Truett *et al.*[18].

DNA measurement: To determine the concentration and purity of the three DNA extraction methods, a NanoDrop 2000 (Thermo Scientific™) was used to measure OD absorption at wavelengths of 260 and 280 nm.

Statistical analysis: Duncan's New Multiple Range Test (DMRT) was used to test variations in DNA concentration and purity using $p<0.05$ as a threshold for significance. Statistical analysis were performed using R version 3.0.2 and the data were modified by additional packages of R.

DNA amplification: DNA extraction from blood and two methods of DNA extraction from the feather calamus were accomplished using MyTaq™ HS Mix (Hot start PCR, Bioline) containing 5 uL of 2x buffer, 2 µL of nuclease-free water and 1 µL of template DNA. In each reaction, four different primer sets were used for amplification by PCR including CHD1F/CHD1R[19] ,2550F/2718R[11], P2/P8[12] and P2/NP/MP[20] (Table 1) using 10 pmol of each primer. The PCR mixture was performed according to the thermal cycling protocol shown in Table 2 with a Biometra TOne Thermal Cycler (Analytik Jena). The PCR products were separated by 2% agarose gel electrophoresis in 0.5x TBE buffer and run at 100 V for 40 min. PCR amplicons were documented under UV light using a camera.

DNA sequencing: PCR amplicons were purified with PCR Clean-Up and Gel Extraction Kit (Bio-Helix) using 300 mg of gel, transferred into a 1.5 mL microcentrifuge tube including 500 µL buffer B and incubated at 60°C for 10 min. The solution was then transferred into a PG column and centrifuged at 13,000 rpm for 30 sec. After that, 400 µL of Buffer W1 was added and the mixture was centrifuged at 13,000 rpm for 30 sec before adding 600 µL of Buffer W2. The mixture was then centrifuged again at 13,000 until excess. Finally, DNA was eluted with 20 µL nuclease-free water at 13,000 rpm for 2 min. All DNA samples were sequenced and visually edited. Base-pair ambiguities were examined using GeneStudio software and compared with published references through the GenBank BLAST application (http://www.ncbi.nlm.nih.gov/BLAST/).

Table 1: Sequences of the four primer sets

Primer set	Primer name	Primer sequence (5-3)
CHD1F/CHD1R	CHD1F	TATCGTCAGTTTCCTTTTCAGGT
	CHD1R	CCTTTTATTGATCCATCAAGCCT
2550F/2718R	2550F	GTTACTGATTCGTCTACGAGA
	2718R	ATTGAAATGATCCAGTGCTTG
P2/P8	P2	TCTGCATCGCTAAATCCTTT
	P8	CTCCCAAGGATGAGRAAYTG
P2/NP/MP	P2	TCTGCATCGCTAAATCCTTT
	NP	GAGAAACTGTGCAAAACAG
	MP	AGTCACTATCAGATCCGGAA

Table 2: PCR program conditions for DNA amplification

	Primer set			
Condition	CHD1F/CHD1R	2550F/2718R	P2/P8	P2/NP/MP
Pre-denaturation	95°C, 5 min	95°C, 5 min	95°C, 5 min	95°C, 5 min
Denaturation	94°C, 30 sec	95°C, 45 sec	95°C, 45 sec	95°C, 45 sec
Annealing	60°C, 30 sec	58°C, 45 sec	56°C, 45 sec	56°C, 45 sec
Extension	72°C, 1 min	72°C, 45 sec	72°C, 45 sec	72°C, 45 sec
Final extension	72°C, 5 min	95°C, 5 min	95°C, 5 min	95°C, 5 min
Cycles	35	35	35	35

RESULTS

Comparison of DNA concentration and purity: Three DNA extraction methods were performed on specimens of red-whiskered bulbul as (1) Blood sample with lysis buffer, (2) Feather calamus with lysis buffer and (3) Feather calamus with alkaline lysis. Results showed that concentrations of DNA extracted using the three methods were statistically different ($p<0.05$) (Fig. 1a). DNA extraction from feather calamus with lysis buffer (II) had the highest concentration ranging from 120-260 ng μL^{-1}, followed by feather calamus with alkaline lysis (III) at 71-128 ng μL^{-1}. DNA extraction from blood samples (I) recorded the lowest DNA concentration, ranging from 43-93 ng μL^{-1}. Purity values (OD260/OD280) of DNA were not statistically different between the three extraction methods (Fig. 1b).

DNA amplification by PCR: For DNA amplification using the PCR technique and separating the size of DNA fragment by 2% agarose gel electrophoresis, results showed that extracted DNA from the three methods could be amplified by 100%, while the four primer sets provided different DNA fragment sizes. The 2550F/2718R primer gave the same DNA fragment size for both males and females, while the three CHD1F/CHD1R, P2/P8 and P2/NP/MP primer sets gave one DNA fragment of the male bird and two DNA fragments of the female bird (Fig. 2). However, the 2550F/2718R and P2/NP/MP

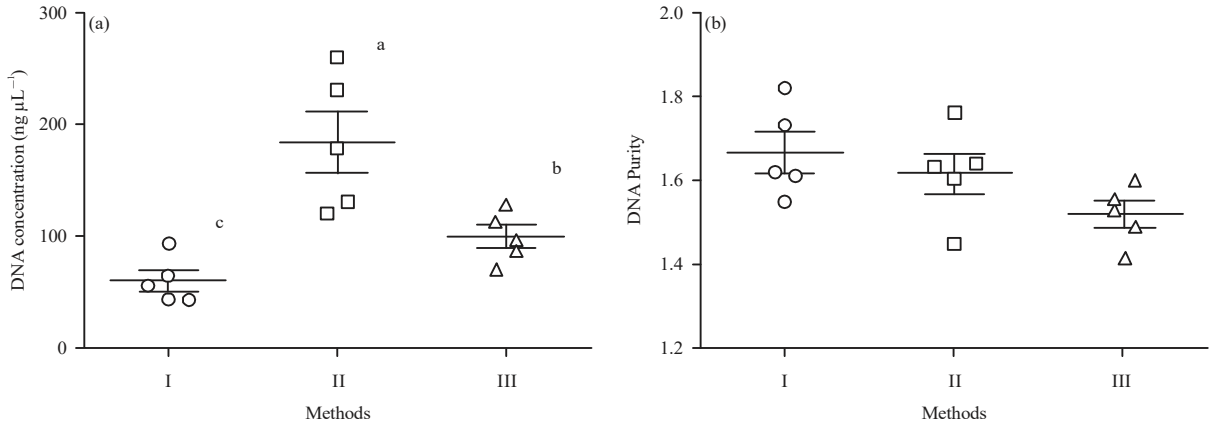

Fig. 1: Means and 95% CI for (a) DNA concentration and (b) Purity. DNA yields obtained from blood samples with lysis buffer method (I), feather calamus with lysis buffer (II) and feather calamus with alkaline lysis (III). Significant differences in DNA extraction methods are indicated by different letters

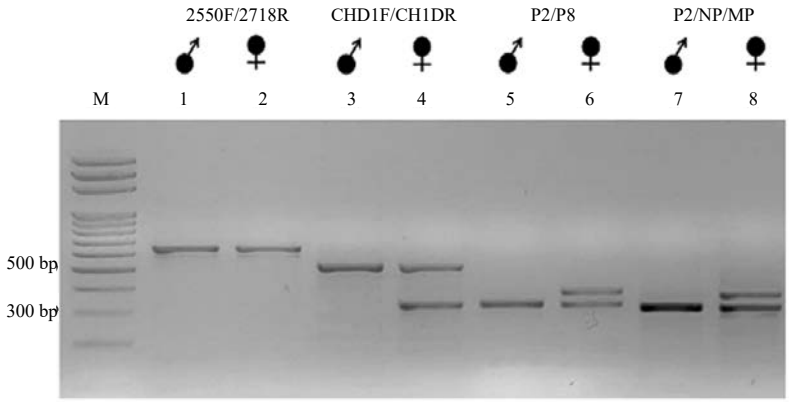

Fig. 2: Sex identification of red-whiskered bulbul using PCR. DNA samples were isolated from the feather calamus with lysis buffer. PCR products of amplified DNA are shown as 2550F/2718R (lanes 1 and 2), CHD1F/CHD1R (lanes 3 and 4), P2/P8 (lanes 5 and 6) and P2/NP/MP (lanes 7 and 8). Lane M shows molecular weight markers (Trackita 1 kb Plus DNA Ladder, Invitrogen). Lengths of marker bands (bp) are indicated to the left

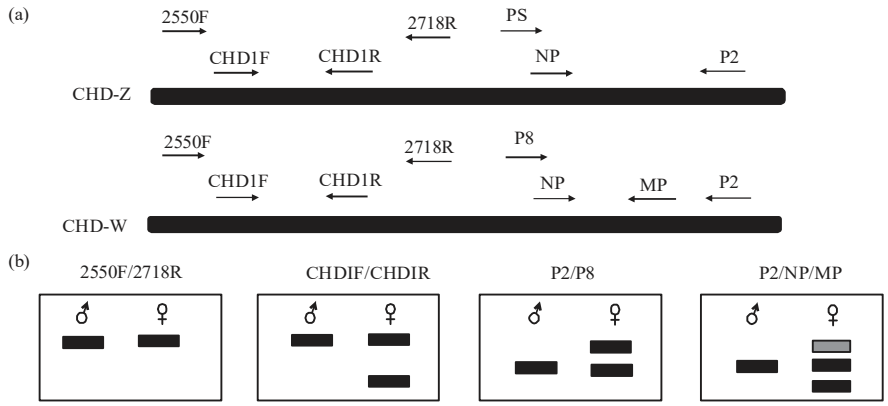

Fig. 3(a-b): (a) Location of primer sets on the CHD-Z and CHD-W genes. Primer sets were aligned with the chicken Z and W chromosomes. (b) Expected DNA fragments from PCR amplification by 255F/271R, CHD1F/CHD1R, P2/P8 and P2/NP/MP. The P2/NP/MP primer had the opportunity to produce triple fragments in females. The gray color bar represents unexpected DNA fragment

Table 3: Homology search of DNA sequences from the four primer sets of PCR products. GenBank accession numbers were used to show the highest identical sequence

Primer	Gene	Size (bp)	Blast result	Identity (%)	Accession No.
2550F/2718R	CHD-Z	654	*Pycnonotus leucogenys*	98.68	HM244403.1
CHD1F/CHD1R	CHD-Z	530	*Pycnonotus leucogenys*	98.49	HM244403.1
	CHD-W	334	*Phylloscopus trochilus*	93.89	AF294633.1
P2/NP/MP	CHD-Z	322	*Pycnonotus jocosus*	99.67	KM058237.1
	CHD-W	368	*Pycnonotus jocosus*	98.92	KM196531.1
P2/P8	CHD-Z	334	*Pycnonotus jocosus*	100.00	KM058237.1
	CHD-W	384	*Pycnonotus jocosus*	100.00	KM196531.1

primer sets did not produce the expected DNA fragments (Fig. 3) obtained from the alignment of primers on chicken CHD-Z (accession number: AC186364.3) and CHD-W (accession number: AC177807.2) genes.

Sequencing analysis: From DNA sequencing, the 2550F/2718R primer set produced only one DNA fragment of male and female birds at 654 bp. The CHD1F/CHD1R primer set produced one fragment in male birds at 526 bp and two fragments in female birds at 526 and 334 bp. The P2/P8 primer set produced one fragment from the male at 334 bp and two fragments 343 and 384 bp in female birds. The P2/NP/MP primer set gave one fragment of male birds at 322 bp and two fragments in female birds at 368 and 322 bp (Table 3). Results showed that primer sets CHD1F/CHD1R and P2/P8 were able to determine the sex of the red-whiskered bulbul as the DNA fragments identified differences between males and females. Nevertheless, the CHD1F/CHD1R primer set more clearly differentiated the PCR product of CHD-Z and CHD-W genes than the P2/P8 primer set (Fig. 2).

The sequence information was compared to the NCBI database. Results showed high homology in related species. CHD-Z and CHD-W partial sequences of the red-whiskered bulbul have been reported in the database of GenBank. Outputs from primers P2/P8 and P2/NP/MP were similar to the database of the red-whiskered bulbul in both CHD-Z and CHD-W genes at more than 98%. Primer set P2/P8 showed 100% homology in both genes. For the CHD1F/CHD1R primer set, output of the CHD-Z gene was close to the DNA sequence of the CHD-Z gene in *P. leucogenys* at 98.49%, while the sequence of CHD-W was similar to the DNA data of the CHD-W gene in *Phylloscopus trochilus* at 93.89%. The 2550F/2718R primer set gave only one fragment which was similar to the CHD-Z gene of *P. leucogenys* at 98.68% (Table 3). The different CHD-Z and CHD-W sequences from amplification by primers CHD1F and CHD1R are shown in Fig. 4.

DISCUSSION

Yields varied in all three methods of DNA extraction depending on technique and sample collection method. The extraction method using lysis buffer consisting of DTT, Proteinase K and SDS gave the highest amount of DNA and purity in the calamus samples. Feather calamus are the source of epithelial and blood cells that can be extracted to yield a

Fig. 4: Alignment of CHD-Z (accession number: MT022017) and CHD-W (accession number: MT022016) gene sequences from DNA samples amplified for CHD1F/CHD1R. Identical and deletion nucleotides between CHD-Z and CHD-W genes are shown with dots (.) and dashes (-), respectively

high amount of DNA. DNA can be extracted at high volume and purity when using the appropriate method[17]. Addition of a chemical such as Proteinase K destroys the protein structure in the feather calamus, while the anionic detergent properties of SDS help to eliminate negative ions in the protein structure. Adding DTT to the lysis buffer can also help to break down the disulfide bond in the protein structure[21]. The method of extracting DNA from the feather calamus using alkaline lysis gave the lowest purity value since the extraction method had no protein removal procedure. One way to digest the cells inside to recover DNA is by using the base properties of NaOH and disodium EDTA to disintegrate the cells. This destabilizes the structure of the phosphodiester bond[22] and can be rectified by adding a neutralizing substance such as Tris-HCl. Although the alkaline lysis DNA extraction techniques gave a low yield of DNA, this method showed high efficiency for bird sex identification. Truett *et al.*[18] reported that the alkaline lysis method can be used to extract DNA for amplifying different products of microsatellites; however, it is not suitable for some applications such as genotyping by southern blot analysis. In the step of DNA digestion, restriction enzymes may undergo interference from substances in the DNA preparation, while DNA may not reanneal efficiently after neutralization.

In general, for sex identification of birds, blood samples are the primary source for DNA extraction. Blood samples extracted with lysis buffer rendered the highest purity compared to other samples. Collecting blood samples using blotting paper can result in a higher purity of DNA. A study of bird blood sample collection using the FTA card method found that extracted DNA was pure and correctly identified the base sequence at 100%, with a higher DNA yield than blood collection by other methods[23]. However, this experiment used the smallest amount of blood required as an invasive technique to extract DNA to reduce injury to the bird. Therefore, the feather calamus presented a better source. Obtaining bird DNA samples for sex determination by PCR can be applied to various biomolecular techniques including finding base sequences or DNA amplification with real-time PCR and LAMP applications.

Four primer sets were used for amplification by PCR to determine the sex of red-whiskered bulbuls. Locations of the primers were defined by alignment on the chicken Z and W chromosomes. The primer patterns were confirmed by comparison with a previous study by Romanov *et al.*[24]. DNA fragments can have two characteristics as (1) The product of the CHD-W gene is smaller than that of the CHD-Z gene such as for primer sets 2550F/2718R[11,25-26], CHD1F/CHD1R[19,27] and P2/NP/MP[20,28-29] and (2) The product of the CHD-W gene is larger than that of the CHD-Z gene such as for the primer set P2/P8[10,12,26].

Results revealed that CHD1F/CHD1R and P2/P8 primers sets could be used to determine the sex of red-whiskered bulbuls whereas primer set 2550F/2718R could not because the DNA fragment produced showed no difference between

males and females. A study of the 2550F/2718R primer found that it could correctly separate sex in 61% of the 49 species tested. However, it cannot be used as a primer in some species due to the location of the CHD-W gene in females. The 2550F/2718R primer cannot, therefore, be complementary in these cases. Avian species that can be sexually identified by the 2550F/2718R primer include the orders Pelecaniformes, Ciconiiformes, Accipitriformes and Phoenicopteriformes (100%), Anseriformes (84.2%) and Passeriformes (3.6%)[13]. In the case of the CHD1F/CHD1R primer, 58 species have been studied for sex identification using the capillary electrophoresis technique to evaluate DNA fragments. DNA fragments from CHD-Z and the CHD-W genes varied from 300-800 bp. The light-vented bulbul (*P. sinensis*), a species related to the red-whiskered bulbul was identified[19]. Corresponding to our results, the CHD-Z gene had approximately 520 bp, with the CHD-W gene at approximately 330 bp. The CHD1F/CHD1R primer set was able to clearly distinguish males and females using the electrophoresis method due to the size difference of approximately 190 bp[19].

Our results showed that the P2/P8 primer set was able to correctly distinguish (100%) the sex of red-whiskered bulbuls. DNA fragments obtained from amplification of the CHD-W gene were larger than the CHD-Z product. DNA fragments of CHD-W are different from CHD-Z 50 bp. However, it is still necessary to use the right amount of agarose gel since the sizes of the CHD-Z and CHD-W genes obtained from the amplification are very similar and they may be combined into a single fragment. Previous studies reported that P2/P8 primers were unable to clearly distinguish the sex of birds because of only 30-50 bp of the CHD-Z and CHD-W genes[30]. The right amount of agarose is more than 2% when studying the effectiveness of P2/NP/MP primers, while the NP primer position was shifted around 10 bp toward the P2 primer and the MP primer was designed at the 3-prime end to be specific to female DNA[20]. The result of the PCR looked different from the product produced by the P2/P8 primer. With regard to the design principle, the MP primer causes an increase in the CHD-W gene in a new location near primer P2, instead of relying on sequence differences in the CHD-Z and CHD-W genes. Therefore, the CHD-W gene product is smaller than the CHD-Z gene[20,28]. Sex identification by P2/NP/MP primers was successful in the orders Passeriformes, Falconiformes and Anseriformes[28] and the family Psittacidae[31]. For gene amplification of this red-whiskered bulbul study, the resulting DNA fragment obtained from the P2/NP/MP series primers did not appear as expected. We found that the DNA fragment had the same characteristics as the P2/P8 primers. Alignment of the P2/NP/MP primers with the nucleotides of the red-whiskered bulbul obtained from DNA sequencing and the GenBank database showed that the MP primer sequence partially matched to the sequence of the red-whiskered bulbul at only 60%. The MP primer was not able to anneal to the DNA template to produce the expected fragment. The resulting output was produced from only P2/NP primers and was similar to the P2/P8 primers since the P8 and NP primers are only slightly different.

CONCLUSION

Feather calamus with lysis buffer for DNA extraction provided the highest DNA concentration, while all three methods for DNA extraction were not statistically different in terms of DNA purity. Primer selections revealed that the 2550F/2718R and P2/NP/MP primer sets were unable to achieve this. Suitable primers were CHD1F/CHD1R and P2/P8. However, the CHD1F/CHD1R primers showed a clearer difference between males and females. Our findings can help to guide methods of sex identification for other species, especially birds in the genus *Pycnonotus*.

SIGNIFICANCE STATEMENT

This study discovered the suitable DNA extraction method and primer for sex identification in the red-whiskered bulbul. The accuracy of sex identification up to 100%. This study can be beneficial for bird breeders in propagation industry for increase mating opportunities on the farm. This study will help the researcher to uncover the critical areas of biomolecular methods for sex identification of birds that many researchers were not able to explore. Thus, a new theory on methods for sex identification of the red-whiskered bulbul may be arrived at.

ACKNOWLEDGMENTS

The authors wish to thank the Faculty of Science, Naresuan University (R2563E016), for financial support. Appreciation is expressed to Mr. Kevin Roebl of the Naresuan University Language Center for constructive comments and English language editing.

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