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Short Communication: Molecular bird sexing on kutilang (*Pycnonotus* sp.) based on amplification of CHD-Z and CHD-W genes by using polymerase chain reaction method

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Abstract. *Pamulang YV, Haryanto A. 2021. Short Communication: Molecular bird sexing on kutilang (*Pycnonotus *sp.) based on amplification of CHD-Z and CHD-W genes by using polymerase chain reaction method. Biodiversitas 22: 449-452.* Sex determination is an important aspect in breeding, ecological studies, rearing and reproductive management on Kutilang (*Pycnonotus* sp.) birds. It is based on differences in intron lengths from DNA amplification of the *Chromodomain Helicase DNA-binding* (CHD) genes on the Z and W sex chromosomes. This study aims to compare the molecular bird sexing method on *Pycnonotus* sp. by PCR amplification derived from two different DNA sources. DNA was extracted from DNA 100 µL peripheral blood (1-2 drops) and three plucked feathers from each sample. The CHD genes were amplified using PCR method and visualized by electrophoresis on 1.5% agarose gel. The amplified DNA fragments were single and double DNA bands in the size of 300 bp for the CHD-Z and 400 bp for the CHD-W genes. Three samples were identified as males from the six Pycnonotus sp. while the other three were females. The molecular sexing of six Kultilang (*Pycnonotus* sp.) birds showed that three samples were males and three were females. Descriptive analysis of two different DNA sources showed that the DNA extracted from peripheral blood samples has better quality than DNA extracted from plucked feathers.

Keywords: CHD gene, kutilang, molecular bird sexing, *Pycnonotus*, PCR

INTRODUCTION

Indonesia has an important role in global biodiversity and one of the 17 countries with mega biodiversity. it has an area of tropical rainforest covering 135 million hectares, and serve as a home to the world's biodiversity consisting of 12% mammals, 16% reptiles and amphibians, and 17% bird species (FAO, 2011). Currently, 1,598 bird species have been identified, of which 372 are endemic to Indonesian, making it first with the largest biodiversity in the world (Rintelen et al. 2017). More than 50% of bird species worldwide are monomorphic (Vucicevic et al. 2013), i.e., there are no significant morphological differences in the external appearance between their males and females. Sex determination is one of the important aspects which serve as a basis for developing breeding strategies, rearing, and reproductive management (Sulandari et al. 2012). One of the problems related to the effort of birds breeding in Indonesia, is the difficulty of phenotypical sex identification of those that are monomorphic in nature, while the dimorphic are more easily phenotypically identified after puberty (Angat and Yusof, 2015). This problem is often found in singing birds from the Passeriformes class, for example, the sooty-headed bulbul or *kutilang* (*Pycnonotus aurigaster*) and the black-crested bulbul or *kutilang emas* (*Pycnonotus melanicterus*) species, which are commonly kept as pets in Indonesia (Mongkolphan et al. 2017).

Sex determination in birds is generally based on behavioral observations, morphometric differences, gonad examination using the laparoscopy or laparotomy method, and the assessment of sex chromosomes. The behavioral observation and morphometry differences gave biased results, while laparoscopy and laparotomy are invasive and risky examinations requiring intensive surgical procedures and post-operative care (Richner, 1989; Dubiec and Zagalskar-Neubauer, 2006). Molecular sex chromosome examination is an alternative method because the procedure is non-invasive and requires a minimal number of samples.

In contrast to mammals, the female birds have heterogametic, namely, W and Z, while males have homogametic, namely Z sex chromosomes (He et al. 2013). The most important in molecular sexing in almost every birds species is the presence of Chromodomain Helicase DNA-binding (CHD) gene which is universal. The sex difference is greatly influenced by the variation of the intron length between the CHD-Z gene in the Z and the CHD-W gene in the W chromosome (Angat and Yusof, 2015). This difference is molecularly analyzed using Polymerase Chain Reaction (PCR) method, in which DNA sequences are amplified, stained and visualized using ultraviolet (UV) light and descriptively assessed (Dubiec and Zagalskar-Neubauer, 2006).

The nucleotide primers pair for amplifying the CHD genes in the molecular bird sexing were designed to anneal between the introns, which makes it possible to

differentiate the PCR amplification products based on the intron length of Z and W sex chromosomes. Male birds generate a single, while the females produce a double band DNA, It due to the PCR amplification in male birds only amplifies introns in the CHD-Z gene, whereas in female birds both introns of CHD-Z and CHD-W genes were amplified which generated the different lengths of PCR products of different lengths (Dubiec and Zagalskar-Neubauer, 2006; Nugroho and Zein, 2015). Various primer pairs have been designed to amplify intron segments of the CHD gene by Griffiths (1998) and Çakmak et al. (2017). P2/P8 primer pair has been used successfully to sex determine the red-whiskered bulbul (*Pycnonotus jocosus*) birds. The design of P2/P8 primers successfully distinguished DNA bands from the amplification products in the CHD-Z and CHD-W genes around 50 bp (Mongkolphan et al. 2017).

Peripheral blood sample is commonly used for DNA-based research in birds. Meanwhile, their erythrocytes have a nucleus that makes it a richer source of nuclear DNA than the blood sample from mammal species (Harvey et al. 2006). Another common bird samples used are plucked feathers. These are widely used because the collection process is not invasive, more convenient, and contain less DNA. In addition, the birds' body that is suitable for the sample collection should be considered, because it interferes with their mobility and orientation of flight (Angat, 2001; Harvey et al. 2006). This study aims to determine the sex of kutilang (*Pycnonotus* sp.) by amplifying the CHD-Z and CHD-W genes on sex chromosomes, and comparing their DNA quality and PCR products collected from the peripheral blood and plucked feathers samples.

MATERIALS AND METHODS

Samples collection

This study involved a total of six kutilang birds, four were Sooty-headed bulbul (*Pycnonotus aurigaster*), and two others were Black-crested bulbul (*Pycnonotus melanicterus*), collected from the Yogyakarta Animal and Ornamental Plants Market (PASTY). The samples from each bird were collected in the form of three plucked feathers. The feather samples were obtained by plucking feathers from the ventral area of the wing, the plucked feathers still must have an intact feather structure, especially the calamus at the apex of the feathers. Blood samples of around 100 µL of peripheral blood (equivalent to 1-2 drops) were taken from the brachial vein, by punching the superficial brachialis vein using a 26-Gauge needle. Then the blood that comes out of the vein is collected into a microhematocrit tube containing heparin.

DNA extraction

The preparation process of feather samples was carried out by cutting into small pieces (0.5-1 cm) of 3 plucked feathers from the apex of feather (calamus) using sterile scissors, the pieces of plucked feathers were collected into a 1.5 mL microcentrifuge, then added by 200 µL GST Buffer and 20 µL Proteinase K. After that the mixture was homogenized with micropastles and incubated for 30 minutes at 60°C. For peripheral blood samples that have been collected into a heparinized microhematocrit tube, were mixed by 200 µL of PBS by spindown process, then the 20 µL of Proteinase K was added and incubated for 30 minutes at 60°C, every 5 minutes the mixture was homogenized by vortex mixer. The further extraction of genomic DNA from lucked feathers and peripheral blood samples was performed in accordance with the standard procedures of Geneaid gSYNC™ DNA Extraction Kit Quick Protocol.

Amplification of CHD-Z and CHD-W genes using the PCR method

The template used in the PCR amplification process was the DNA extracted from the peripheral blood and calamus of the plucked feather taken from the *Pycnonotus* sp. birds. The DNA amplification was targeted on the Chromodomain Helicase DNA-binding (CHD) genes of the Z and W sex chromosomes using a primer pair consisting of P2-F as the forward and the P2-R as the reverse, as reported by Griffiths (1998). Detailed information on the nucleotide primer pairs was presented in Table 1.

The PCR mix with a total volume of 25 µL comprised 12.5 µL of MyTaq HS Red Mix DNA polymerase, 1 µL (10 pmol) for each primer (P2-F and P2-R) and 9.5 µL of DNA template. The amplification in the thermocycler consisted of several stages, starting from pre-denaturation at 94°C for 2 mins, followed by denaturation at 94°C for 20 secs, annealing at 46°C for 30 secs, elongation at 72°C for 40 secs, and finally post-elongation at 72°C for 10 mins. The denaturation, annealing and elongation stages were repeated for 40 cycles.

DNA electrophoresis on agarose gel

The results of the DNA amplification products were visualized by electrophoresis method using 1.5% agarose gel with 2 µL SYBRsafe DNA staining in 1x Tris/Borate/EDTA (TBE) buffer solution. The agarose gel was electrophoresed by subjecting it to an electric current of 80 Ampere and 100 volts for 35 mins. The resulted DNA bands were visualized by SYBRsafe staining and observed under ultraviolet light.

Table 1. Nucleotides sequence, annealing temperature (Ta) and melting temperature (Tm) of P2-F and P2R primers for the amplification of the CHD genes (Nugraheni et al. 2019; Khaerunnisa et al. 2013)

Primer	Base sequence	∑Base	Ta (°C)	Tm (°C)
P2-F	5'-CTCCCAAGGATGAGRAAYTG-3'	20	46	52,8
P2-R	5'-TCTGCATCGCTAAATCCTTT-3'	20	46	51,9

RESULTS AND DISCUSSION

The results of the DNA extraction from peripheral blood and plucked feather samples of six kutilang (*Pycnonotus* sp.) birds were electrophoresed on 1.5% agarose gel as presented in Figure 1.

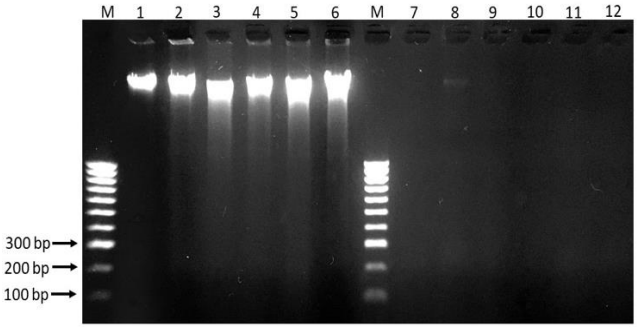

Figure 11. Total extracted DNA from 2 different types of samples. M was Hyperlader DNA Markers 100 bp (M). No, 1-6 were extracted DNA templates from peripheral blood samples. No. 7-12 were extracted DNA template from plucked feather of *Pycnonotus* sp.

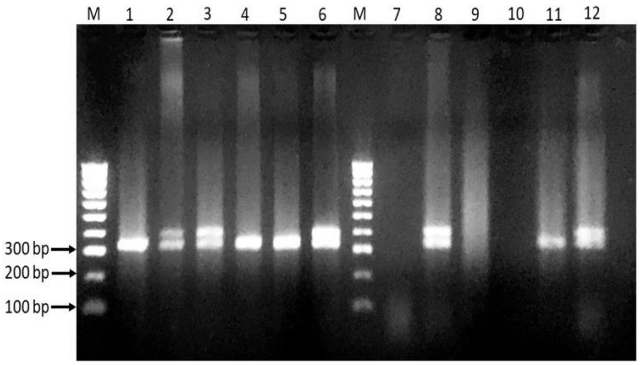

Figure 2. Electrophoresis gel showing polymerase chain reaction product for the CHD genes of birds *Pycnonotus* sp. Hyperladder marker 100 bp (M). The amplification results of kutilang (*Pycnonotus* sp.) blood samples (No. 1-6) and the amplification results of kutilang (*Pycnonotus* sp.) feather samples (No 7-12).

Table 2. Bird sexing interpretation based on the number of band from PCR products

Sample code	Electrophoresis result	Interpretation
1	Single band	Male
2	Double band	Female
3	Double band	Female
4	Single band	Male
5	Single band	Male
6	Double band	Female
7	-	-
8	Double band	Female
9	-	-
10	-	-
11	Single band	Male
12	Double band	Female

Figure 1 showed that the results of the DNA extraction from peripheral blood samples were shown in well number 1-6 which appeared as thick and clear fluorescent bands when visualized under the UV light. The results of DNA extraction from plucked feather samples shown in well number 7-12. Samples 7, 9, 10, 11, and 12 did not show any clear DNA band luminescence, while 8 produced a thin fluorescent type. The extracted DNA electrophoresis results were in line with the previous study conducted by Harvey et al. (2006), stating that the isolated peripheral blood of birds contained more DNA compared to plucked feathers. Also, it contains more cell nuclei than the plucked feathers since it is only derived from the epithelial cells in the calamus stuck at the end of the feathers. In addition, the low quality of DNA in plucked feather samples is caused by the presence of keratin, inhibitor protein that interfere in the process of DNA extraction. A single plucked feather consists of more than 90% beta keratin, 8% water, 1% lipids, and 1% protein (Khaerunnisa et al. 2013). The Hyperladder markers 100 bp in the first and eighth wells produced fluorescent DNA bands in sizes of 100 bp to 1,000 bp.

The total genomic extracted DNA was used as template for PCR amplification by pair of primers P2 and P8. Nine samples from the total of twelve were successfully PCR amplified and visualized on 1.5% agarose gel electrophoresis as presented in Figure 2.

As shown in Figure 2, DNA samples no. 2, 3, and 6 from peripheral blood and plucked feathers sample no.8 and 12. generated double DNA bands in size of 300 bp and 400 bp it showed female sample. In contrast, male *Pycnonotus* sp. only generated a single DNA band in size of 300 bp. The peripheral blood (No. 1, 4, and 5) and a plucked feather (No. 11) generated a visible and clear single DNA band, while other feather samples (No. 7, 9, 10) produced a thin band which was not identified clearly. The bird sexing interpretation of *Pycnonotus* sp. based on the band number of PCR products was further interpreted in Table 2. It showed that three samples from the six *Pycnonotus* sp. were female birds and the other three were males. These results were concurrent with the study of Nugraheni et al. (2019), which determined bird sexing on Peach-faced Lovebirds (*Agapornis roseicollis*), and Argarini et al. (2020) on Fischeri Lovebirds (*Agapornis fischeri*) using PCR techniques.

In this study, set primer P8 as primer forward dan P2 as primer reverse were used for PCR amplification of CHD genes of *Pycnonotus* sp. These primers were designed by Griffiths et al. (1998), which have been reported to successfully identified sex of 11 different bird orders, including Passeriformes. However, these primers have never been reported to be used as sex determinants in sooty-headed bulbul (*Pycnonotus aurigaster*) and black-crested bulbul (*Pycnonotus melanicterus*).

P8 and P2 primers were a specific set of nucleotide which was designed to anneal on exon regions of the bird CHD genes, both on CHD-W in the W and CHD-Z in the Z sex chromosome. These primers pair amplified exon and intron regions of the CHD genes. In the PCR amplification process, P8 or P2-F primers anneal to the CHD genes' exon

and amplified the exon and intron forward (forward amplification), while P2 or P2-R primers amplified the CHD genes in the opposite or reverse direction (reverse amplification).

In Figure 2, female *Pycnonotus* sp. birds showed double DNA bands in sizes of 300 and 400 bp, while males only indicated a single DNA band in the size of 300 bp. According to Griffiths et al. (1998), in most bird species, the CHD-W amplicon in females has a larger size. CHD-Z amplicon is used as a control because this gene is always amplified in either male or female birds. It was shown in Figure 2 that the CHD-W and CHD-Z amplicon were 400 bp and 300 bp. The difference of PCR product length into agarose gel electrophoresis was similar to the previous study, as reported by Dubiec and Zagalskar-Neubauer (2006), in which the difference of DNA amplicons between CHD-W and CHD-Z using P8 and P2 primers set was in range of 10 – 80 bp.

The agarose electrophoresis of PCR products in Table 2 and Figure 2 showed that three feather samples No. 7, 9, and 10 did not generate DNA bands that were clearly observed although the PCR optimization has been carried. The unclear PCR products were possibly caused by low quality of DNA or not enough concentration of the template in the amplification process. This indicated that the DNA template from the feather samples had a very low concentration. The factor that caused the difference in plucked feather samples from each individual was different amount of calamus collected from the feathers (Purwaningrum et al. 2019). Larger feather samples were deeper in the bird's body and contain more DNA from epithelial cells (Peters et al. 2019).

In conclusion, the molecular sexing of six Kultilang (*Pycnonotus* sp.) birds showed that three samples were males and three were females. Descriptive analysis of two different DNA sources showed that the DNA extracted from peripheral blood samples has better quality than DNA extracted from plucked feathers.

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