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Short Communication

Molecular Bird Sexing of Tanimbar Cockatoos (*Cacatua goffiniana*) by Using Polymerase Chain Reaction Method

Ratu Fresa Khoirotunnisa Hidayat[1], Diana Savitri[1], Irhamna Putri[2], Warih Pulung Nugrahani[2], Aris Haryanto[1]*

1) Department of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta. Jl. Fauna 2, Karangmalang, Yogyakarta 55281, Indonesia.
2) Wildlife Rescue Centre (WRC), Jl. Pengasih-Nanggulan, Pengasih, Kulon Progo, Yogyakarta 55652, Indonesia.
* Corresponding author, email: arisharyanto@ugm.ac.id

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ABSTRACT

This study aimed to determine the sex of Tanimbar Cockatoo (*Cacatua goffiniana*) birds by amplifying Chromodomain Helicase DNA-binding-1 (CHD-1) gene on Z and W sex chromosomes as well as to compare the quality of DNA extraction and PCR amplification products from samples derived from peripheral blood and plucked feathers. This work used five *C. goffiniana* birds which were collected from the Wildlife Rescue Center (WRC) in Pengasih, Kulon Progo, Yogyakarta. From each *C. goffiniana*, feather samples were collected by plucking feathers on the ventral wing and peripheral blood samples were taken by cutting their nails and collecting the blood into microhematocrit tubes containing heparin. The next stage was DNA extraction and DNA amplification on the CHD-1 gene using the PCR method by NP, P2, and MP primer pairs. Then, products of DNA extraction and PCR amplification were electrophoresed on 1.5% agarose gel and visualized under a UV light transilluminator with a wavelength of 260 nm. The visualization showed that samples from peripheral blood generated clearer DNA fragments compared to plucked feathers. Two of the five samples were male *C. goffiniana* and the other three samples were females. In the male Tanimbar Cockatoo was amplified a single DNA fragment of the Z sex chromosome in size of 300 bp, whereas in the female *C. goffiniana* was amplified double DNA fragments of Z and W sex chromosomes in size of 300 bp and 400 bp respectively. The DNA quality showed that the DNA quality from peripheral blood samples were better in quality than the DNA collected from plucked feather samples.

Keywords: *Cacatua goffiniana,* CHD-1, molecular bird sexing, PCR, sex chromosome

Indonesia is known as a country with abundant biodiversity, both flora and fauna, especially various species of birds. Indonesia is the fourth country after Columbia, Peru and Brazil with the number of bird species. Indonesia has 1.598 species of birds, including 372 (23.28%) endemic bird species, 149 (9.32%) species of migratory birds and 118 (7.38%) species of endangered birds (Kurniawan et al. 2018). These birds need to be protected and preserved in order to avoid extinction. One species of endangered bird is *Cacatua goffiniana*, which is also called Tanimbar Cockatoo, Kakatua Tanimbar, or Corella Tanimbar because it is an endemic bird from the

Tanimbar island in Southeast Maluku. Its habitat is in dry tropical forests and humid deciduous forests (Mioduszewska et al. 2018). In 1992, Tanimbar Cockatoo was included in the Appendix 1 category by the Convention on International Trade in Endangered Species (CITES) because it was on the verge of extinction, so this bird was banned to be traded in all forms of international trading (Jepson et al. 2001). The declining population has raised the conservation status of *Cacatua goffiniana* to near threatened (BirdLife 2018). The threat was identified as a result of habitat loss and illegal hunting. They lost their habitat because of forest fires to create more agricultural fields and fewer nesting sites due to logging of Manilkara fasciculate and *Canarium indicum* trees (Mioduszewska et al. 2018).

Tanimbar Cockatoo has distinctive characteristics of white feathers and a white crest with red patches on the feathers around the beak. Its body size is about 320 mm and it weighs 350 grams (Zein et al. 2017). This bird is rather small compared to other cockatoo species. The uniqueness of the Tanimbar Cockatoo is its loud voice and its intelligence in combining image objects (Habl & Auersperg 2017). Its beauty and uniqueness have led to illegal hunting of this bird to be traded so that the population is endangered. Efforts in preserving the population of Tanimbar Cockatoo include improving the ecological environment, biological evolution, breeding, and conservation. Determining the sex of the bird quickly and accurately also supports these efforts. (Ravindran et al. 2019).

Tanimbar Cockatoo is a monomorphic bird, in which male and female birds have the same physical characteristics until they are mature so it is difficult to distinguish them morphologically. Determination of sex in monomorphic bird groups can be done using several methods such as laparoscopy, karyotyping and vent sexing. Laparoscopic and karyotyping methods have weaknesses; they are expensive and require a long time, while the weakness of the vent sexing method is that the results are less accurate and can only be done during the breeding season. Other methods that can be used are morphometric analysis and molecular bird sexing. Morphometric analysis has the advantages of being fast and inexpensive as well as applicable outside of the breeding season, but it has a low confidence level if there is no other supporting data. Molecular bird sexing has several advantages, namely it is accurate, applicable for any age of the bird, and relatively safe. This method has been widely applied to groups of parrots (Psittacidae). However, the weakness of this method is that it can only be done in a laboratory and is relatively expensive (Liu et al. 2011; Kurniawan & Arifianto 2017).

Molecular bird sexing is a DNA-based sex determination that is most commonly applied to wild birds. Samples used to obtain genetic material can derive from peripheral blood or plucked feathers. The blood of birds has nucleated erythrocytes that are rich in DNA. On the other hand, a plucked feather has less DNA and is easily degraded if it originates from dead cells such as mature feathers. However, the advantage of plucked feather samples is that they are easy to obtain, reduce the risk of contamination and avoid pain so that the costs required are also lower (Harvey et al. 2006). Determination of sex by molecular bird sexing involves sex chromosomes in the Chromodomain Helicase DNA-binding-1 (CHD-1) gene. The sex chromosomes of birds are ZW hemizygous chromosomes in female birds and homozygous ZZ chromosomes in male birds. The CHD-1 gene on the Z and W sex chromosomes have different intron sizes. This can be used as a basis for determining the sex of birds by amplifying the CHD-1 target gene by using the Polymerase Chain Reaction (PCR) method. The PCR method requires specific primer pairs that are designed to amplify the two exon areas in a sequence of nitrogenous bases that are similar in many bird species and are located between introns. The results of amplification in male birds

produce only a single DNA fragment from the Z sex chromosome, whereas in female birds the amplification produces double DNA fragments from the Z and W sex chromosomes (Liu et al. 2011; Nugroho & Zein 2015).

Primer pairs used for amplification of the CHD-1 gene intron segment have been extensively developed by researchers. The intron segment of the CHD1 gene is used as a marker of sex determination because it has significant size differences between the sexes (Nugroho & Zein 2015). The P2/P8 primers design which was developed by Griffiths et al. (1998) cannot distinguish between male and female birds. Fridolfsson and Ellegren (1999) have developed a 2550F/2718R primers design that is able to overcome problems of P2/P8 primary pairs. The latest development states that birds from the Psittaciformes order such as Conure, Parrot, and Macaw have been able to be tested for their sex by using NP, P2, and MP primers which result in clearer differences between male and female birds. NP primer is a forward primer that will attach to the CHD-1Z and CHD-1W genes, while a reverse primer consisting of two primers, P2 and MP, will attach to the CHD-1Z gene and the CHD-1W gene (Thammakam et al. 2007). This study aimed to determine the sex of the Tanimbar Cockatoo bird by amplifying the CHD-1 gene on the Z and W sex chromosomes, as well as comparing the quality of extracted DNA and PCR amplification products from samples derived from peripheral blood and samples derived from plucked feathers.

The research samples were plucked feathers and peripheral blood from five Tanimbar Cockatoo (*Cacatua goffiniana*) birds collections of the Wildlife Rescue Center (WRC), Pengasih, Kulon Progo, Daerah Istimewa Yogyakarta. The feather samples were taken from the ventral wing. The peripheral blood samples were taken by cutting the nails, and then the blood was kept in microhematocrit containing heparin anti-coagulant. The samples were sent to the Biochemistry Laboratory of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta. The samples were coded CD1, CD2, CD3, CD4, and CD5 for samples derived from peripheral blood and CB1, CB2, CB3, CB4, and CB5 codes for samples derived from plucked feathers.

The procedure used for DNA extraction from feather and peripheral blood samples was carried out following the standard procedure from Geneaid gSYNC™ DNA Extraction Kit Quick Protocol.

The results of DNA extraction are used as a template for the DNA amplification process using the PCR method. DNA fragments were amplified with the CHD-1 gene target on the Z and W sex chromosomes by using specific primer pairs, namely P2-Forward, NP-Reverse, and MP-Reverse primers. The composition of the nucleotide sequence, number of bases, references of the three primers are presented in Table 1.

The master mix composition for PCR amplification with a total volume of 25 µl consists of 12.5 µl MyTaq HS Red Mix DNA polymerase, 1 µl P2 primer, 1 µl MP primer, 1 µl NP primer, and 9.5 µl DNA template. The mixture was mixed until homogeneous and then transferred into a thermocycler with a process consisting of the pre-denaturation stage at a temperature of 94 °C for 2 minutes, denaturation at a temperature of 94 °C for 20 seconds, annealing at a temperature of 46 °C for 30 seconds, elongation at a temperature of 72 °C for 40 seconds and post-elongation at a

Table 1. Nucleotide sequence of P2, NP, and MP primers for amplification of CHD1 gene.

Primer	Nucleotide Sequence	Σ Base	Reference
P2	5'-TCTGCATCGCTAAATCCTTT-3'	20	Griffiths et al. 1998
MP	5'-AGTCACTATCAGATCCGGAA-3'	20	Ito et al. 2003
NP	5'-GAGAAACTGTGCAAAACAG-3'	19	Ito et al. 2003

temperature of 72 °C for 10 minutes. The denaturation, annealing, and elongation stages were repeated in 40 cycles.

DNA electrophoresis was carried out to visualize the results of DNA extraction and the results of PCR amplification with ultraviolet (UV) transilluminators. Agarose gel was used for electrophoresis at a concentration of 1.5% in a 1x TBE solution supplemented with 2 µl SYBRsafe DNA staining. Electrophoresis of all samples in 1.5% agarose gel was carried out at a voltage of 100 volts and an electric current of 80 mA for 45 minutes. Electrophoresis results were observed with an ultraviolet (UV) transilluminator with a wavelength of 260 nm in a dark room.

Electrophoresis of total DNA extraction and CHD-1 gene amplification results observed by using UV-Transilluminator in a dark room are presented in Figures 1 and 2.

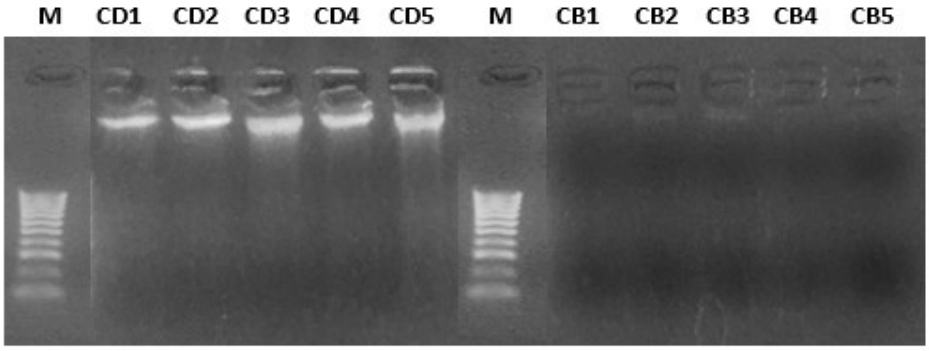

Figure 1. Electrophoresis of total DNA extraction. Note: M = marker (hyperladder 100 bp), CD1-CD5 = peripheral blood samples of Tanimbar Cockatoo, CB1-CB5 = feather samples of Tanimbar Cockatoo (*Cacatua goffiniana*).

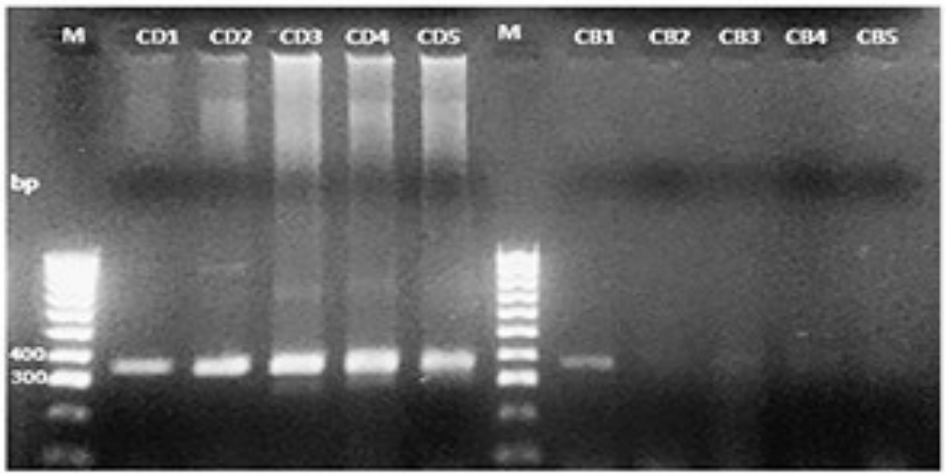

Figure 2. Electrophoresis of CHD-1 gene amplification of samples from Tanimbar Cockatoo (*Cacatua goffiniana*). Note: M = marker (hyperladder 100 bp), CD1-CD5 = *C. goffiniana* peripheral blood samples, CB1-CB5 = *C. goffiniana* feather samples.

Based on the electrophoresis results in Figures 1 and 2, a comparison of the quality of the DNA extraction results. Here, we have compared the purity of DNA extracted from peripheral blood and plucked feather samples did not perform quantitatively using a UV spectrophotometer, but performed descriptively based on Figures 1 and 2. These Figures showed that the extracted DNA and PCR products derived from peripheral blood samples appeared clearer, cleaner and there was no smear DNA band compared to the samples from plucked feathers. The products of PCR amplification of peripheral blood and plucked feather samples were more detailed presented in Table 2.

Table 2. Comparison of extraction and amplification results between *Cacatua goffiniana* blood and feather samples.

No	Bird Code	Extraction Results		Amplification Results	
		Blood	Feather	Blood	Feather
1.	*C. goffiniana* 1	+++	+	+++	++
2.	*C. goffiniana* 2	+++	+	+++	+
3.	*C. goffiniana* 3	+++	+	+++	+
4.	*C. goffiniana* 4	+++	+	+++	+
5.	*C. goffiniana* 5	+++	+	+++	+

Note: +: not really clear, ++: quite clear, +++: clear

Visualization of the results of DNA amplification by PCR method in the *C. goffiniana* CHD-1 gene was further interpreted with the results presented in Table 3.

Table 3. Interpretation of the visualization of PCR amplification.

No.	Sample Code	Electrophoresis Result	Interpretation
1.	CD1	Single band	Male
2.	CD2	Single band	Male
3.	CD3	Double bands	Female
4.	CD4	Double bands	Female
5.	CD5	Double bands	Female
6.	CB1	Single band	Male
7.	CB2	Not really clear	-
8.	CB3	Not really clear	-
9.	CB4	Not really clear	-
10.	CB5	Not really clear	-

The electrophoresis results in Figure 1 showed that DNA fragments are seen in peripheral blood samples with the sample code CD1, CD2, CD3, CD4, and CD5, while feather samples with the sample code CB1, CB2, CB3, CB4, and CB5 looked empty and do not display the DNA band. This indicates that the DNA in the feather samples was either very thin or non-existent, so it cannot be visualized properly in the agarose gel 1.5% by SYBRsafe staining. According to Harvey et al. (2006), DNA extracted from feather samples was less in quantity compared to DNA extracted from peripheral blood. The results of total DNA extraction in Figure 1, are then used as a template for PCR amplification with the CHD-1 gene target on the Z and W sex chromosomes using NP, P2, and MP primer pairs.

PCR is an enzymatic method for in vitro DNA amplification. This method is used to multiply DNA fragments millions of times in a short time with the use of the enzyme DNA polymerase and a pair of primers specific to the target DNA. Specific primers used in this study included 3 primers, namely NP, P2, and MP primers developed by Ito et al. (2003) as a substitute for P2/P8 primers that were not able to detect differences in CHD-1 genes intron on Z and W chromosomes so that they cannot distinguish between male and female birds. Out of 29 bird species in East Asia, NP, P2 and MP primers can identify 25 bird species belonging to seven different orders namely Passeriformes, Falconiformes, Columbiformes, Strigiformes, Gruiformes, Ciconiiformes, and Caprimulgiformes (Lee et al. 2008). According to Thammakam et al. (2007), the primers were also successfully used to determine the sex of birds in Psittaciformes order such as Conure,

Parrot, and Macaw. In addition, NP, P2, and MP primers were able to determine 100% of samples from 14 Rosy-faced Lovebirds (*Agapornis roseicollis*) (Nugraheni et al. 2019). Other similar studies also showed that the sex of 54 birds in captivity originating from 11 different families can be determined by using these primers (Purwaningrum et al. 2019) and Pamulang and Haryanto (2021) who successfully carried out a molecular bird sexing on kutilang (*Pycnonotus aurigaster* and *Pycnonotus melanicterus*) birds by using PCR amplification on CHD-Z and CHD-W targeted genes.

The PCR amplification results of the CHD-1 gene on Tanimbar Cockatoo (*Cacatua goffiniana*) in Figure 2 showed that male birds only generated a single DNA fragment from the Z sex chromosome, whereas female birds generated double DNA fragments from Z and W sex chromosomes (Nugroho & Zein 2015). NP, P2 and MP primers were used for amplification of introns in the CHD-1Z and CHD-1W gene segments. NP primer was a forward primer that attaches to the CHD-1Z and CHD-1W genes, while the reverse primer consists of two primers: P2 and MP primers. P2 primer will attach to the CHD-1Z gene and MP primer will attach to the CHD-1W gene (Thammakam et al. 2007). Electrophoresis results in Figure 2 showed that the peripheral blood samples produce good and clear DNA bands in both male and female *C. goffiniana*, whereas the feather samples produce thin DNA bands. The electrophoresis of DNA extraction results cannot be seen clearly, so the DNA cannot be visualized properly. In addition, DNA extraction results from feather samples appear to be less in quantity and more easily degraded compared to DNA extracted from peripheral blood samples (Harvey et al. 2006). Another factor that caused the thin DNA bands in the feather samples was the different amount of calamus collected in the five samples. According to Purwaningrum et al. (2019), feather samples with more number of calamus produce clearer DNA bands in the electrophoresis.

Comparison of DNA extraction results and PCR amplification results in Table 2 showed that peripheral blood samples were observed to be the better source of DNA template in molecular bird sexing by PCR method compared to feather samples. According to Harvey et al. (2006), bird blood has nucleated erythrocytes that are rich in DNA. Therefore, only a few drops of blood were needed for the DNA extraction process. However, the collection of blood samples was more difficult because birds have relatively small blood vessels that can cause stress that may lead to death. The blood samples in this study were collected by cutting one of the nails of the Tanimbar Cockatoo, which was then collected on a microhematocrit containing anti-coagulant heparin. This method was chosen to minimize the risk of stress in Tanimbar Cockatoo. Feather samples for determining the sex of Tanimbar Cockatoo were taken from the ventral wing by plucking the feather up to the base of the hair (calamus). Calamus is a source of DNA that contains many living epithelial cells or blood deposits in the feather shaft. According to Harvey et al. (2006), a feather sample produces less quantity of DNA compared to blood samples, and the DNA was easily degraded which makes the both the quality and quantity of DNA were lower. Ravindran et al. (2019) also state that blood samples were more accurate than feather samples as DNA sources. Extraction of feather samples produces a smaller amount of DNA because there was a keratin protein in the calamus that can act as an inhibiting protein in the DNA extraction process. (Hickman et al. 1984).

Based on the interpretations presented in Table 3, it can be seen that from the five Tanimbar Cockatoo (*Cacatua goffiniana*) blood samples coded as CD1, CD2, CD3, CD4, and CD5 there were two male Tanimbar Cockatoos (CD1 and CD2 samples), while the other three (CD3, CD4, and CD5 samples) were female Tanimbar Cockatoos. Male sex was characterized by

the production of a single DNA fragment of 400 bp from the Z sex chromosome, whereas females were characterized by the appearance of double DNA fragments of 300 bp and 400 bp from the W and Z sex chromosomes (Nugroho & Zein 2015). A single DNA fragment indicating male sex was produced in one feather sample (CB1 sample), while CB2, CB3, CB4, and CB5 feather samples produced thin and unclear bands that could not be interpreted.

Molecular bird sexing of five Tanimbar Cockatoo (*C. goffiniana*) birds showed that two samples were male *C. goffiniana* with an amplification product of a single DNA band in size of 400 bp from the Z sex chromosome, while three samples were female *C. goffiniana* because generated double DNA bands in the size of 300 bp from the Z sex chromosome and 400 bp from the W sex chromosome respectively. This finding was in line with other researchers (Savitri et al. 2021), who have successfully carried out molecular bird sexing on Sulphur-crested Cockatoo (*Cacatua galerita*) by PCR amplification. The quality of extracted and PCR amplified DNA showed that the DNA quality from peripheral blood samples were better in quality than the DNA collected from plucked feather samples.

AUTHOR CONTRIBUTION

RFKH carried out laboratory works (samples collection, DNA extraction, PCR amplification, agarose gel electrophoresis, data analysis, and drafted the manuscript). AH planned and designed the research work as well as translated and revised the final manuscript. DS got permission for field research conducting. IP and WPN handling and collected field samples. All authors contributed to this research and approved the final manuscript.

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CONFLICT OF INTEREST

The author declares that there is no competing interest

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