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# Molecular bird sexing of sulphur-crested cockatoo (*Cacatua galerita*) by polymerase chain reaction method

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**ABSTRACT** Sex identification of endangered and protected birds in captivity is very important for conservation programs. Half of the world's bird species are monomorphic, where male and female are difficult to distinguished morphologically, including cockatoos. Sex identification using molecular bird sexing is more accurate and applicable because it directly targets the sex chromosomes. The purpose of this study was to determine the sex of Sulphur-crested cockatoo (*Cacatua galerita*) by detecting differences in the intron size of the chromodomain helicase DNA-binding 1 (*CHD1*) gene on the Z and W chromosomes by polymerase chain reaction (PCR) method and to compare of plucked feathers and blood samples as DNA sources for molecular bird sexing. DNA was extracted from feather and blood samples from four *C. galerita*. Extracted DNA was amplified on the *CHD1* gene by PCR method with P2, MP, and NP primers, which were visualized using agarose gel 1.5% under UV transilluminator with a wavelength of 280 nm. The resulting PCR product was detected at 392 bp for the *CHD1* Z gene segment and 297 bp for *CHD1* W gene segments, where males showed a single DNA band (ZZ) and females showed a double DNA band (ZW). Four *C. galerita* were 100% successfully determined, consisting of one female and three males. Electrophoresis results showed DNA bands from blood samples were thicker and brighter than DNA bands from feather samples.

**KEYWORDS** *CHD1* gene; conservation; sex identification; Z and W chromosomes

## 1. Introduction

Sulphur-crested cockatoo (Ca*catua galerita*) is a type of cockatoo found in Eastern Indonesia, Papua New Guinea and Australia (BirdLifeInternational 2019). *C. galerita* is also known as the yellow-crested big cockatoo because of its similarities with *Cacatua sulphurea*, but *C. galerita* has a bigger body with a body length of 380-510 mm, a wingspan of more than 260 mm, and bodyweight of approximately between 815 to 975 g (Beehler et al. 2001; WorldParrotTrust 2018). This bird has white feathers all over its body, except its yellow crest, the underside of the wings, and tail feathers. It has a black and very compact beak and blue eye circles (Foreshaw 2010; Rowley and Kirwan 2016).

*Cacatua galerita* is one of the curved beak birds commonly kept as a pet by Indonesian since its docile nature and ability to imitate human voices. The rise of illegal capture of this bird can be threaten its conservation in nature (Nugroho and Zein 2015). Indonesian government classifies *C. galerita* as a protected animal and to maintain its conservation, the Indonesian government has issued several regulations, including Law No. 5 Year 1990 concerning Conservation of Living Resources and Their Ecosystems, Government Regulation No. 7 Year 1999 on Preserving Flora and Fauna Species, and Regulation of the Minister of Environment and Forestry No. 106 Year 2018 regarding Second Amendment to Regulation of the Minister of Environment and Forestry No. 20 Year 2018 regarding Types of Protected Plants and Animals. Conservation status of *C. galerita* in International Union for Conservation of Nature (IUCN) is Least concern (LC) (BirdLifeInternational 2019). Although *C. galerita* is classified as the LC status, the distribution number of its wild population is decreasing. According to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), *C. galerita* is included in the appendix II category (Hay 2008) and not an endangered animal, however, the trade of these animals should be regulated to prevent excessive trading (Beno and Ohee 2009). Preservation of cockatoos is essential for conserving the population and prevent extinction.

One of the attemps to preserve cockatoos is the bird conservation activities, including *in situ* developing natural habitats, and *ex situ* breeding outside of their natural

habitat. Ability to distinguish the sex of the cockatoos in captivity is an important aspect that should be considered in breeding for the prospective broodstock and registering the data of the chicks (Nugroho and Zein 2015). *C. galerita* are monomorphic birds, where male and female birds have almost the same morphology, size and color, so that distinguishing the sex by using observation alone is rather difficult (Rowley and Kirwan 2016). Various methods are developed for bird sexing, such as karyotype, hormone analysis, laparoscopy, and vent sexing. However, these methods have various disadvantages, such as being time-consuming, high cost, invasive and difficult to apply in the field, or can only be carried out on a certain period of time. Other methods for bird sexing are the molecular method and morphometric analysis method (Dubiec and Zagalska-Neubauer 2006; Cerit and Avanus 2007).

The molecular method, developed by (Fridolfsson and Ellegren 1999), is a method for determining the sex of birds based on deoxyribonucleic acid (DNA), which is based on the differences among the intron size of the Chromodomain Helicase DNA-binding 1 (*CHD1*) gene in the Z and W chromosomes. Molecular method expedites identification of a bird's sex since it can be applied in the bird as young as 5-7 days; and take for short determination (Purwaningrum et al. 2019). Aves has heterozygous ZW sex chromosomes in females and homozygous ZZ sex chromosomes in males (Grant 2001). The *CHD1* gene was discovered to exist in both Z (*CHD1*-Z) and W (*CHD1*-W) chromosomes in most ratite bird species with different lengths of introns, thus utilization of *CHD1* as a genetic marker is suitable for sex determination (Griffiths and Korn 1997; Ellegren 1996). The *CHD1* gene is amplified by using the polymerase chain reaction (PCR) method using several primers, such as 2550 F and 0718 R as well as P2 and P8 primer set which successfully performed for sex determination in 58 bird species and evaluation of CHD gene as a universal molecular marker in bird sexing by Vucicevic et al. (2013). The amplicon of DNA fragment from the Z chromosome in male birds and two DNA fragments in female birds from Z and W chromosomes are generated. There are different sizes length of DNA fragments due to differences in the length of the amplified intron (Fridolfsson and Ellegren 1999; Liu et al. 2011). Over the past two decades, the study on sex determination using molecular methods is gradually developed since it is considered highly accurate, relatively practical, non-invasive, and fast in determining sex in young birds and monomorphic birds (Nugroho and Zein 2015). It has been reported that the application of *CHD1* gene for sex determination of avian is a non-invasive technique for sex determination of monomorphic birds (Angat and Yusof 2015; Khaerunnisa et al. 2013).

## 2. Materials and Methods

### 2.1. Ethical approval

This study complies with the ethical requirements of the Ethical Clearance Commission of the Faculty of Veterinary Medicine, Universitas Gadjah Mada (Approval no. 0013/EC-FKH/Int./2020), as well as local laws and regulations.

### 2.2. Specimen collection

The samples used in this study were plucked feathers and a few drops of blood from four Sulphur-crested Cockatoo (*C. galerita*) from the Yogyakarta Wildlife Rescue Center (WRC) in Wates, Yogyakarta. The blood samples were coded as B1, B2, B3, and B4 and the feathers samples were coded as F1, F2, F3, and F4. The plucked feather samples were collected by plucking three to five feathers (secondary flight feathers) in the ventral part of the wings. The blood samples were taken by cutting the birds's nails near the blood vessels to reduce stress, and then the blood was collected in microhematocrit containing heparin anticoagulant. Feather and blood samples were stored in airtight plastic containing silica gel for further processing at the Laboratory of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Universitas Gadjah Mada.

### 2.3. DNA extraction

About 0.5 to 1 cm from three to five feathers (1 g), including the calamus of each sample, was cut. Theprocedure for DNA extraction from feathers followed the standard procedure on the Geneaid gSYNC™ DNA Extraction Kit (Taiwan) Quick Protocol.

### 2.4. Amplification of CHD1 gene by PCR

The extracted DNA (0.38 µg/µl) was used as a template in the amplification process using the PCR method. DNA fragments were amplified by targeting the *CHD1* gene on the sex chromosome using P2 (Griffiths et al. 1998), NP and MP primers (Ito et al. 2003). The sequences of nucleotides on P2, NP, and MP primers are presented in Table 1.

The amplification process was carried out with a total reaction volume of 25 µl with composition details based on the kit protocol MyTaq$^{TM}$ (Bioline UK). The amplification reaction was carried out using a Thermal Cycler (Cleaver Scientific GTC96S) machine with an optimal PCR condition for amplification of DNA fragments, i.e.

**TABLE 1** The nucleotide sequences of P2, NP, and MP primers to amplify the *CHD1* gene.

| Primer | Basic sequence | Σ Base | Tm (°C) |
|---|---|---|---|
| P2-R | 5'-TCTGCATCGCTAAATCCTTT-3' | 20 | 56 |
| MP-R | 5'-AGTCACTATCAGATCCGGAA-3' | 20 | 58 |
| NP-F | 5'-GAGAAACTGTGCAAAACAG-3' | 19 | 54 |

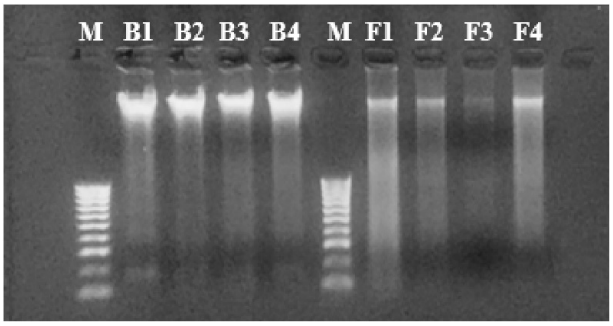


**FIGURE 1** Electrophoresisr result of the total DNA extraction of Sulphur-crested Cockatoo (*Cacatua galerita*) samples. Note: M = marker (hyperladder 100 bp), B1-B4 = blood samples, F1-F4 = feather samples.

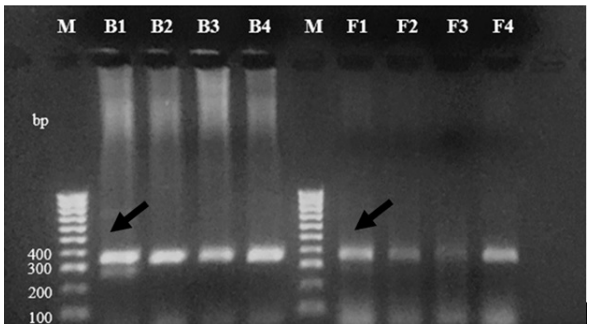


**FIGURE 2** Electrophoresis result of *CHD1* gene amplification samples from Sulphur-crested Cockatoo (*Cacatua galerita*). Note: M = marker (hyperladder 100 bp), B1-B4 = blood samples, F1-F4 = feather samples, arrow = double band samples.

pre-denaturation at 94 °C for 2 min, denaturation at 94 °C for 20 s, annealing or primer attachment at 46 °C for 30 s, extension at 72 °C for 40 s, and post-extension at 72 °C for 10 min. The denaturation, annealing, and extension stages were repeated in 40 cycles.

The resulting PCR was visualized in agarose electrophoresis. The concentration of the agarose gel was 1.5%. PCR amplification to be observed had a length of 297 bp for single band, and 297 bp and 392 bp for two bands.

## 3. Results and Discussion

Electrophoresis from total DNA extraction and DNA amplification from *CHD1* gene were analyzed by UV transilluminator. The electrophoresis results in the agarose gel are presented in Figure 1 and Figure 2.

Based on Figures 1 and 2, blood samples and feather samples can be compared. The results of DNA extraction and the results of PCR amplification of the CHD gene were observed. The comparison is presented in Table 2 below.

The interpretation of the electrophoresis of the amplification resultof the *CHD1* gene segment in blood and plucked feather samples of *C. galerita* is presented in Table 3.

The genome DNA was clear and thick in blood samples coded B1, B2, B3, and B4. In the plucked feather samples, namely F1, F2, F3, and F4, the genome DNA is clear but thinner than those ofthe blood samples. It shows less genome DNA extracted from the plucked feather sample so that it is not visualized maximally. These results are consistent with the previous report by Harvey et al. (2006) that the amount of DNA from feather samples is lower than DNA from blood samples. The extraction results were then used as a template to amplify the *CHD1* gene on Z and W chromosomes by using the PCR method using P2, NP, and MP primers.

The molecular method for bird sex determination in this study is based on the PCR result, which is a method of in vitro amplification or multiplication of DNA molecules in specific areas bounded by a pair of primers. Amplification of the *CHD1* gene was performed on a thermal cycler with annealing temperature of 46 °C for 40 cycles. Determination of sex in birds with P2, NP, and MP primers can be visualized under UV transilluminator at a wavelength of 280 nm. Male birds will produce one DNA band while female birds will produce two DNA bands. According to Morinha et al. (2012), aves have sex chromosomes that are different from mammals. In birds, the heterogeneous nature is found in the females (ZW) while the males are homogeneous (ZZ). The relationship between the position of the *CHD1* gene and sex chromosomes in aves (Z and W chromosomes) causes the *CHD1* gene to show differences in Z and W alleles in females (Griffiths and Korn 1997).

Ito et al. (2003) reported that the design of the P2, NP, and MP primers was based on differences in sequences between *CHD1* W and *CHD1* Z genes using 3'-terminal mismatch primer on point mutation of the female *CHD1* W gene. In contrast to mammals, female birds have heterogametic sex chromosomes, namely W and Z sex chromosomes, while male birds have homogametic sex chromosomes, namely Z sex chromosomes. In each species of

**TABLE 2** Comparison of the DNA extraction and amplification of the *CHD1* gene segment between *Cacatua galerita* blood samples and plucked feathers.

| Bird Code | Result of DNA Extraction | | Result of CHD1 Amplification | |
|---|---|---|---|---|
| | Blood | Feather | Blood | Feather |
| *Cacatua galerita* 1 | +++ | ++ | +++ | ++ |
| *Cacatua galerita* 2 | +++ | ++ | +++ | ++ |
| *Cacatua galerita* 3 | +++ | + | +++ | + |
| *Cacatua galerita* 4 | +++ | ++ | +++ | ++ |

**TABLE 3** Interpretation of the results of *CHD1* Gene Segment Amplification.

| Sample Code | Number of band | Interpretation |
|---|---|---|
| B1 | Two bands | Female |
| B2 | One band | Male |
| B3 | One band | Male |
| B4 | One band | Male |
| F1 | Two bands | Female |
| F2 | One band | Male |
| F3 | One band | Male |
| F4 | One band | Male |

bird, molecular sexing universally carried out by detecting the presence of the Chromodomain Helicase DNA-binding (*CHD*) gene. PCR amplification with the CHD gene targeted can be used to detect sex differences of birds due to the difference of intron length between the *CHD1* Z gene on the Z sex chromosome and the *CHD1* W gene on the W chromosome sex chromosome (Angat and Yusof 2015).

A set of designed primer pairs will anneal at the position between the introns of the CHD gene. It is possible to differentiate the PCR amplification products based on the length and number of PCR products generated from the Z and W sex chromosomes. In male birds generated PCR products in the form of single band DNA in size of 392 bp. whereas in female birds generated double band DNA in size of 297 bp and 392 bp (Dubiec and Zagalska-Neubauer 2006). According to Garofalo et al. (2016), NP is a forward primer while P2 and MP are reverse primers. The P2 primer will attach to the *CHD1* Z gene on the Z chromosome, while the MP primer will attach to the *CHD1* W gene on the W chromosome and only one fragment (Z) appears in males, while two fragments (Z and W) appear in females because they have different lengths of band due to differences in the length of the amplified introns.

Based on the electrophoresis of results, the *CHD1* gene can be used to distinguish the sex of *C. galerita* (Figure 2). Previously, *CHD1* gene can be used to distinguish the sex of family Cacatuidae, i.e. *Cacatua sanguinea*, *Probosciger aterrimus*, and *Eolophus roseicapi* (Purwaningrum et al. 2019). The expected size of PCR products was 297 bp for the W chromosome and 392 bp for the Z chromosome. Ito et al. (2003) reported that using P2, MP and NP primers, all of the male bird samples produced 392 bp bands in the *CHD1* Z gene segment amplification, while the female birds would produce two 392 bp and 297 bp PCR product bands for *CHD1* Z and *CHD1* W gene segments.

Blood samples B1, B2, B3, and B4 showed very thick and clear bands, making it easier to distinguish between males and females. Feather samples F1, F2, and F3 produced thinner and fairly clear bands. The electrophoresis results in total DNA extraction appeared to be thin, while F4 produces thick and clear bands. The clearness of the DNA band in the feather samples is determined by the number of calamus, where the samples with more calamus show a clearer DNA band when electrophoresed (Purwaningrum et al. 2019).

The comparison table (Table 2) shows that blood samples are better sources of DNA for molecular bird sexing compared to plucked feather samples. Birds' blood has nucleated erythrocytes thus, it contains a lot of nucleus DNA. The source of DNA in feathers is obtained from the basal portion of the feather (calamus) containing a lot of epithelial cells, but a protein inhibitor named keratin makes the extraction process more difficult and it produces a lower quantity of DNA compared to blood (Hickman et al. 1984). Research conducted by Harvey et al. (2006) and Ravindran et al. (2019) showed similar comparison results where blood samples were more accurate as a source of DNA than extracted feather samples in molecular bird sexing. As seen in Table 3, the four Sulphur-crested Cockatoo (*C. galerita*) are identified as one female bird and three male birds.

Molecular sex identification in birds is usually performed by using plucked feather and blood samples. Other kinds of samples, such as cloaca swabs, buccal swabs, and feces, can also be used, but they require a longer collection time. Blood samples are collected from the jugular vein, brachial vein (wing), or medial metatarsal vein (depending on species and age of the bird) and stored in capillary tubes containing anticoagulants. Feather samples are collected by pulling bird feathers on the ventral part of the wing or chest (Dubiec and Zagalska-Neubauer 2006).

Since the nucleated bird erythrocytes are a source of abundant DNA, only a few blood drops are needed for the DNA extraction process. Harvey et al. (2006) reported that blood samples produce more DNA compared to feathers, but collecting blood samples from birds requires more energy. Improper collection of blood samples can cause stress to the bird. The blood sample in this study was collected by cutting the nails close to blood vessels in order to reduce stress in birds, and then the blood was collected in microhematocrit containing heparin anticoagulant.

The use of feathers as samples has the advantage of providing accurate (the accuracy equal to sample from blood), safe and non-life threatening results. It is also quick, inexpensive, and easy to collect the feathers. DNA samples obtained from feather samples cause less stress on birds and thus more considerate towards the animal welfare (Cerit and Avanus 2007). According to Cakmak et al. (2017), feather samples produce less DNA compared to blood samples. Hence, DNA produced from feather samples is more easily degraded so that the quality and quantity of DNA is low. However, by considering the speed, ease, and minimal risk, feathers are chosen as samples of molecular bird sexing (Harvey et al. 2006). The feather samples used in this study were collected by plucking the feathers on the ventral part of the wings.

## 4. Conclusions

Visualization of the results CHD1 gene segment amplification showed that of four Sulphur-crested Cockatoo (*C. ga-*

*lerita*) tested, there were three males and one female with an intron size of 297 bp on the W chromosome and 392 bp on the Z chromosome. Visualization of DNA extraction results and amplification of blood samples is thicker and clearer than the feather samples, so that blood samples are a better and more accurate source of DNA in molecular bird sexing than feather samples.

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## Authors' contributions

AH planned and designed the study. MP took care of the research permits. IP and WPN collected samples. DS and AH carried out the work (DNA extraction, PCR examination, DNA electrophoresis). DS drafted the manuscript; AH translated and revised the manuscript. All authors contributed to this research and approved the final manuscript.

## Competing interests

The author declare that they have no competing interest.

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