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Short Communication

Molecular Bird Sexing of Small Yellow-crested Cockatoo (*Cacatua sulphurea,* Gmelin 1788) Using Polymerase Chain Reaction Method

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ABSTRACT
The yellow-crested cockatoo (*Cacatua sulphurea,* Gmelin 1788) is an endemic bird in eastern part of Indonesia with monomorphic characteristics and included in the list of endangered birds. A method of sex determine in monomorphic birds is by molecular sexing which is based on the PCR amplification of the *chromodomain helicase DNA-binding 1* (CHD-1) gene of the bird sex chromosome. This study was aimed to sex determine of the *C. sulphurea* by amplifying the CHD-1 gene on the Z and W chromosomes and comparing the PCR amplification results from peripheral blood and plucked feathers samples. The samples used were four birds of *C. sulphurea* from the Wildlife Rescue Centre (WRC), Yogyakarta. The feathers obtained from the ventral wings of each bird were plucked. Through the cutting of the birds' nails, the peripheral blood samples were collected into microhematocrit tubes which contained Heparin. The amplification of the CHD-1 gene used the PCR method with specific primers, such as NP, P2, and MP. Moreover, the PCR results were visualized on 1.5% agarose gel using UV-Transilluminator, at a wavelength of 280 nm. The PCR products (amplicons) were in 297 bp and 392 bp DNA bands, depending on the sex of the bird being tested. It was also observed that the male *C. sulphurea* produced single 392 bp DNA fragment of the Z chromosome. However, the female birds produced two DNA fragments of the Z and W chromosomes, with a length of 297 bp and 392 bp. The results showed that samples obtained from peripheral blood produced clearer DNA bands compared to plucked feather. It concludes that the extracted DNA from peripheral blood samples have a better quality compared to samples from plucked feathers. Amplification of the CHD-1 gene in male *C. sulphurea* generated only a single DNA fragment in size of 392 bp, so the four *C. sulphurea* were male birds.

More than 35,000 wild animals in the world have been categorized as endangered species (CITES 2015), including a variety of birds. The main causes of these extinctions are the destruction of natural habitats and illegal hunting. Moreover, wildlife trade has been observed to be a serious threat to the survival of animals in nature. According to reports, about 95% of traded species originated from natural hunting with only 5% from captivity (Zein et al. 2017). One of the birds experiencing such population decline is the small

yellow-crested Cockatoo (*Cacatua sulphurea*). This species is one of the endemic birds in the eastern part of Indonesia, with other habitats ranging from Nusa Penida island to the Wallacea bioregion area. The economic value of this bird has led to increased hunting, due to the great demand from collectors or bird hobbyists (Widhiantara et al. 2016).

The number of illegally captured cockatoos continued to increase, as it is being reported to be a threat to the survival of these birds in nature. Therefore, the Ministry of Environment and Forestry of the Republic of Indonesia is campaigning for the cockatoo rescue program, where people are urged to voluntarily release the illegally captured birds to the government. This rescue service is likely to be accompanied by a programwhich enables the release of these birds into their natural habitat. The process of releasing the cockatoos that have lived with humans for a long time requires several stages, in order for them to possess the abilities to live naturally in the wild environment. These stages involved are as follow: (1) Identification of each individual bird. (2) Preparation of the bird's condition for proper release. (3) Release of birds into their natural habitats by the proper distribution process. (4) Monitoring the birds after being released to the wild environment. Since these cockatoos are to be returned to the distribution area of their original habitat; phenotypic and genotypic studies are needed to determine the species; lineage and sex of these birds (Zein et al. 2017).

The use of molecular technology for sex determination is also reported to be beneficial for conservational efforts and the matchmaking process of birds, e.g., the small yellow crested cockatoo (*C. sulphurea*). These cockatoos are classified as monomorphic birds, with males and females having similar physical characteristics, making them difficult to be phenotypically differentiated. Several methods that have been used in determining the sex of birds include the vent and steroid sexing, laparoscopy, and karyotyping. Even though these methods have been widely used to determine the sex of monomorphic birds, they still possess several weaknesses, such as being expensive and invasive, as well as consuming a large amount of time (Morinha et al. 2012). Moreover, molecular bird sexing has been applied to various species, especially in the Psittacidae family (curve-billed birds). This method has been reported to have the advantage of being non-invasive, as it less threatens the safety and depends on the age of the birds, while it is also providing faster and more accurate results (Kurniawan & Arifianto 2017).

Furthermore, molecular bird sexing is known to be the simplest and most widely used method of sex determination, based on DNA amplification. A wide variety of specific nucleotide primers pairs to amplify intron segments of the Chromodomain Helicase DNA-binding I (CHD-1) gene, has also been reported to be used. This intron segment amplification is also used as a marker for sex determination, due to having a significant difference in the size of the amplified DNA, for both male and female birds (Morinha et al. 2012). Also, the analysis of this sex determination was based on size differences of the CHD-1 gene's amplification results on the Z and

W chromosomes, via the use of the PCR method. This amplification also has the ability to multiply the target genes in most monomorphic birds, by producing single and double fragments of DNA amplicon in male (Z chromosome) and female (Z and W chromosomes) species, respectively (Fridolfsson & Ellegren 1999; Nugroho & Moch 2015). Moreover, this molecular sexing by amplification of CHD-gene on the Z and W chromosomes have also been successfully performed by researchers on other monomorphic and Kutilang birds, such as Peach-faced Lovebird (*Agapornis roseicollis*) (Nugraheni et al. 2019), with Sooty-headed and Black-crested bulbuls (*Pycnonotus aurigaster & Pycnonotus melanicterus*) (Pamulang & Haryanto 2021). The aim of this study was to determine the sex of the yellow crested cockatoo (*C. sulphurea*) on the Chromodomain Helicase DNA-binding I (CHD-1) target gene in the Z and W chromosomes, via the use of the PCR method. It also aimed to compare the quality of isolated and produced DNA from the PCR amplification derivatives of two different samples, namely peripheral blood and plucked feathers.

The samples in this study were collected from peripheral blood and plucked feathers of 4 small yellow-crested cockatoos (*C. sulphurea*), which were obtained from the Wildlife Rescue Centre (WRC) collection, in Kulon Progo, Yogyakarta. Preparations of these samples were carried out at the Biochemistry Laboratory, the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta. The peripheral blood samples were coded SD1, SD2, SD3 and SD4, with that of the plucked feathers being SB1, SB2, SB3, and SB4.

This research met the ethical requirements of both the Ethical Clearance Commission of the Veterinary Medicine Faculty, Universitas Gadjah Mada, Yogyakarta (Approval no. 0013/EC-FKH/Int./2020), and local laws regulations.

According to the standard procedures of the Geneaid GSYNC™ DNA Extraction Kit Quick Protocol, DNA isolation from the samples was performed. This genetical isolation from the peripheral samples was carried out by collecting blood in a microhematocrit tube and plucked feather samples was performed by removing two or three primary feathers from *C. sulphurea*. The results of the DNA isolation from both peripheral blood and calamus feathers of *C. sulphurea* were then used as genetic templates in the amplification process, via the use of the PCR method. These DNA templates were then amplified by targeting the CHD-1 encoding gene on the Z and W chromosomes, by the use of the specific nucleotide primer pairs P2, NP, and MP which have been designed by Griffiths et al. (1998) and Ito et al. (2003). The nucleotide sequence, number of bases in each primer, with the annealing temperature (Tan) and melting temperatures (Tm) based on Thammakarn et al. (2007), they were presented in Table 1.

The compositions of the PCR reagent mixture in one reaction of a 25 µL total volume (consisted MyTaq™ DNA Polymerase), P2, NP, and MP

primers, and the DNA template isolated from both peripheral blood and feather calamus samples, were presented in Table 2.

Table 1. Nucleotide sequence, annealing temperature (Ta) and melting temperature (Tm) of P2, NP, and MP primers for amplification of the CHD-1 gene.

Primer	Nucleotide Sequence	∑ N Base	Tan (°C)	Tm (°C)
NP-F	5'-GAGAAACTGTGCAAAACAG-3'	19	46	49.5
P2-R	5'-TCTGCATCGCTAAATCCTTT-3'	20	46	41.9
MP-R	5'-AGTCACTATCAGATCCGGAA-3'	20	46	52.3

Furthermore, the mixture was placed into a thermocycler, with the conditions for the temperature and duration of the PCR reaction as follows, (1) Pre-denaturation at 94°C for 2 mins, (2) Denaturing at 94°C for 20 secs, (3) Annealing at 46°C for 30 secs, (4) Elongation at 72°C for 40 secs, (5) Post -elongation at 72°C for 10 mins. Moreover, repetition of the denaturation, annealing, and elongation stages were still carried out in 40 cycles. This PCR condition was performed according to Savitri et al. (2021), who has performed a molecular bird sexing by PCR on sulphur-crested cockatoo (*C. galerita*).

Furthermore, the extracted DNA from the samples (peripheral blood & plucked feathers) and PCR products were also electrophoresed in agarose gel with a concentration 1.5% in a 1x Tris-Buffered-EDTA (TBE) supplemented with 2 µL SYBR Safe DNA staining.

Afterwards, eight DNA samples (4 peripheral blood and 4 plucked feather samples) were ready to be loaded in the 1.5% agarose gel. In each of these samples, 8 µL of DNA was collected and added with 2 µL loading dye, as 4 µL of Hyperladder 100 bp DNA. The voltage was set to 100 volts with an electric current at 80 mA, for 45 mins running duration. The agarose gel electrophoresis of extracted DNA and PCR products was then observed on the UV-Transilluminator, with a wavelength of 280 nm. This DNA electrophoresis method was performed according to Argarini et al. (2020), who have a molecular bird sexing on fischeri lovebird (*Agapornis fischeri*) by using PCR amplification.

Table 2. Compositions of PCR reagents mixtures for yellow-crested Cockatoo (*C. sulphurea*) DNA in one reaction for the CHD-1 gene.

Sample Code	MyTaq™ DNA Polymerase (µl)	Forward Primer NP (20 pmol/µl) (µl)	Reverse Primer P2 (20 pmol/µl) (µl)	Reverse Primer MP (20 pmol/ µl) (µl)	DNA Template (100 ng/µl) (µl)	Total Volume (µl)
SD1	12.5	1	1	1	9.5	25
SD2	12.5	1	1	1	9.5	25
SD3	12.5	1	1	1	9.5	25
SD4	12.5	1	1	1	9.5	25
SB1	12.5	1	1	1	9.5	25
SB2	12.5	1	1	1	9.5	25
SB3	12.5	1	1	1	9.5	25
SB4	12.5	1	1	1	9.5	25

The DNA was successfully extracted from the four samples of the peripheral blood and plucked feathers of *C. sulphurea* birds. The extracted DNA was then used as a template for PCR amplification, by targeting the Chromodomain Helicase DNA-binding-1 (CHD-1) gene on the Z and W chromosomes, via the use of specific nucleotide primers pairs, namely NP, P2, and MP. The results of the electrophoresis extracted DNA from the peripheral blood and plucked feathers of *C. sulphurea,* were presented in Figure 1.

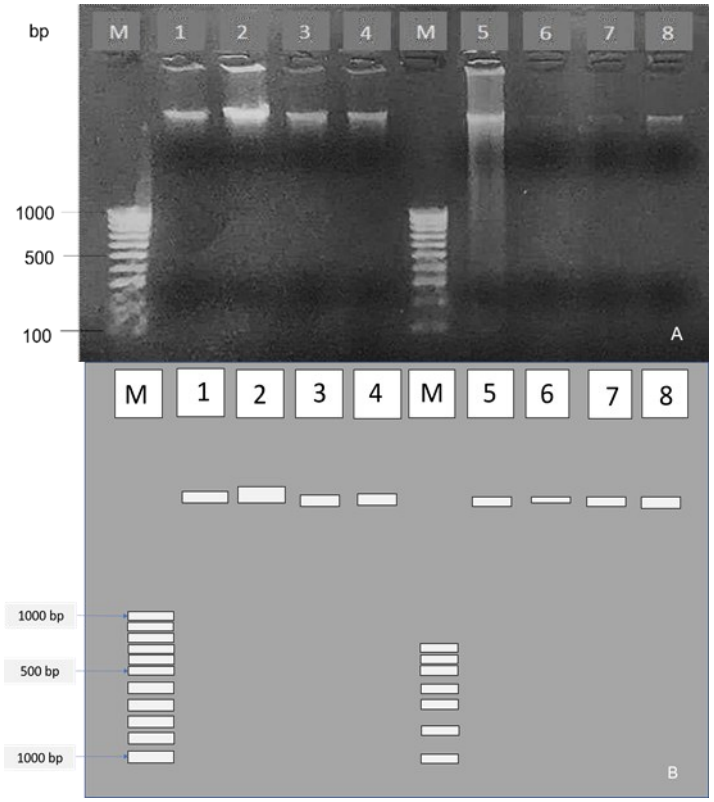

Figure 1. A. Electrophoresis of total extracted DNA. Note: M = DNA marker (hyperladder 100 bp), 1-4 = blood samples of *C. sulphurea*, 5-8 = plucked feather samples of *C. sulphurea*. B. Electrophoregram of Figure 1A.

Comparisons of the amplified CHD-1 gene electrophoresis with the 100 bp Hyperladder DNA Marker, which were observed on the UV-Transilluminator, resulted in products (PCR) in the form of DNA bands, with a length of 297 bp and 392 bp, respectively. The results of this PCR products electrophoresis on 1.5% agarose gel, were presented in Figure 2.

Sample number 1, 2, 3, 4 and 5 produced a fairly clear DNA bands, with values 6, 7 and 8 exhibiting a very thin display. Due to this result, the visualization of the PCR amplification of the CHD-1 gene in these samples was difficult to observe. Generally, the results of DNA electrophoresis showed that samples from peripheral blood produced a clearer image of DNA bands, compared to the thin display obtained from the plucked feathers. Furthermore, the electrophoresis of PCR amplified CHD-1 gene (from blood samples of *C. sulphurea* birds) compared with control samples obtained from the male and female *Cockatoo sp.,* was presented in Figure 3.

Moreover, the visualization results of the amplified CHD-1 gene in samples 1, 2, 3, and 4, showed only single DNA band, which was in size of 392 bp. The electrophoresis of PCR products in Figure 2 and 3 showed the results that were in line with Bosnjak et al. (2013), who have studied the feasibility of non-invasive molecular methods for sexing of Parrots.

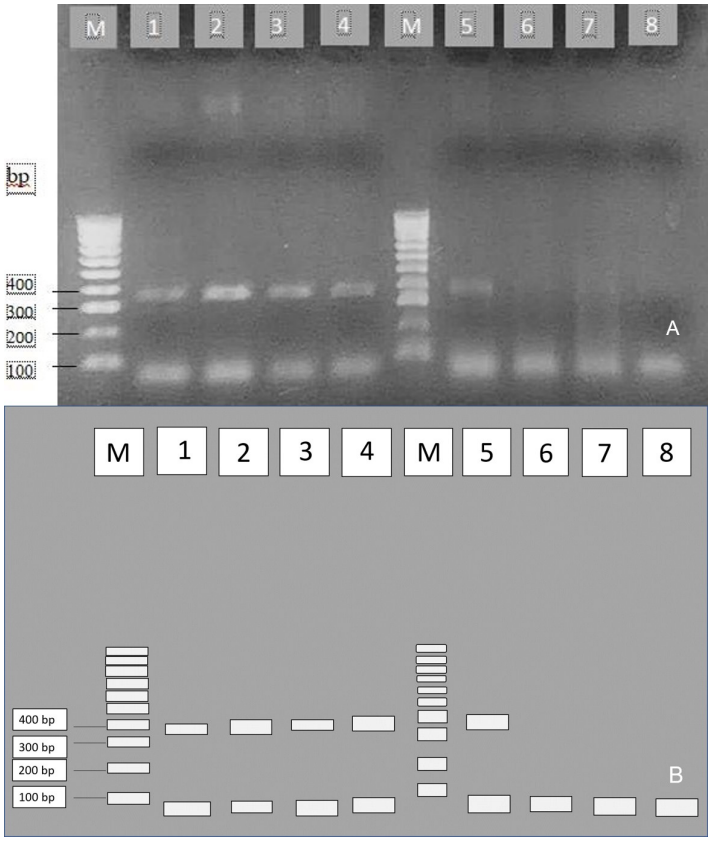

Figure 2. A. Electrophoresis of the amplified CHD-1 gene from samples taken from *C. sulphurea* bird. M= hyperladder 100 bp DNA marker, lane no. 1-4 = peripheral blood sample of *C. sulphurea*, lane no. 5-8 = plucked calamus samples of *C. sulphurea*. B. Electrophoregram of Figure 2A.

It was well known that extracted DNA from peripheral blood has better quality than extracted DNA from lucked feather. However to sex determine of endangered birds, a non-invasive method is required, because the invasive sample collection method has a high risk for species of endangered birds, therefore, the extracting DNA method from plucked feathers taken still needs to be improved as have been successfully performed in Avian by Khaerunnisa et al. (2013).

Table 3. Comparison between the quality of extracted DNA and PCR amplified DNA from peripheral blood samples and plucked feather samples from *C.sulphurea*.

No	Sample Code	Extraction		PCR Amplification	
		Blood	Plucked Feather	Blood	Plucked Feather
1.	***C. sulphurea* 1**	+++	++	+++	++
2.	***C. sulphurea* 2**	+++	+	+++	+
3.	***C. sulphurea* 3**	+++	+	+++	+
4.	***C. sulphurea* 4**	+++	+	+++	+

Note: +++: clear, ++: clear enough, +: less clear.

Based on Table 3, the comparison of the quality between extracted and PCR amplified DNAs indicated that the genetic origination from peripheral blood samples was better, compared to those obtained from the plucked feathers. Also, it indicated that blood sample was a better source of DNA in molecular bird sexing. Remedios et al. (2010) also stated that blood sample was an effective source of genetic template for molecular bird sexing, due to the fact that erythrocytes in birds had a nucleus which made them rich sources of DNA. The source of DNA in plucked feathers was only obtained from the calamus, which contained inhibitory protein (keratin) that made extraction process more difficult, with less quantity of DNA being obtained (Hickman et al. 2004).

The results of the DNA extraction also showed that the bands from the blood samples were visualized clearly, compared to those from the feathers. In Figure 1, samples with clearly visible DNA bands indicated that the quantity of extraction was numerous, as visualization was significant. However, samples with thin DNA bands indicated that the quantity of extraction was very little, as visualization was not clear enough.

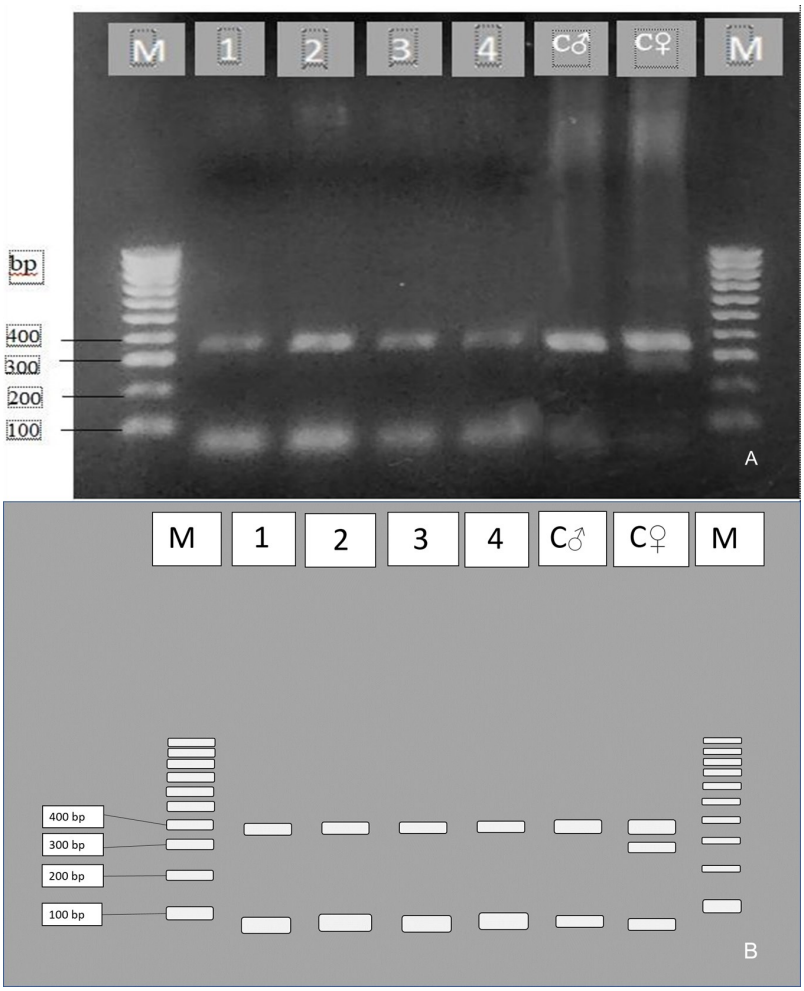

Figure 3. A. Electrophoresis of CHD-1 gen amplification result of *C. sulphurea* peripheral blood samples compared to control male and female cockatoos. M = Hyperladder 100 bp DNA marker, Lane 1-4 = peripheral blood sample of *C. sulphurea*; C♂ = control male *Cacatua sp*; C♀ = control female *Cacatua sp*. B. Electrophoregram of Figure 3A.

In this study, DNA amplification was observed to have used the PCR method, which targeted the CHD-1 gene on the W and Z chromosomes in *C. sulphurea*. This PCR was an *in vitro* reaction, which was used to multiply the number of DNA molecules on a particular gene. Moreover, this reaction was carried out by using a specific nucleotide primer pair, which complemented the target DNA that was to be amplified. However, this reaction occurred with the help of the DNA polymerase enzyme. The specific primers used in this study were the NP, P2, and MP, which were developed by Ito et al. (2003). These primers were used as a substitute for the those often used, namely P2 and P8, which were developed by Griffiths et al. (1998), due to the fact that they were unable to detect differences in the introns of the CHD gene on the Z and W chromosomes. The results of a study conducted by Lee et al. (2008), showed that out of the 29 species that tested, NP, P2, and MP, the primers only succeeded in determining the sex of 25, which belonged to seven different orders, namely Ciconiformes, Falconiformes, Gruiformes, Columbiformes, Strigiformes, Caprimulgiformes, and Passeriformes. A research by Thammakarn et al. (2007) also observed that these primers were used to determine the sex of several bird species, such as conures, macaws, and parrots, which belonged to the order Psittaciformes. Use of three primers (NP, P2, MP) for sex determination of *C. sulphurea*, was that these primer pairs have been successfully performed to sex determine by other researchers with other types of Cacatua birds, as reported in previous study by Savitri et al. (2021) to sex determine of *C. galerita* as well as by Hidayat et al. (2021) for *C. goffiniana.*

Also, the amplification of the CHD-1 gene was carried out *in vitro* with a thermocycler, at the optimized annealing temperature of 46°C for 30 secs, in 40 cycles. Sex determination with NP, P2, and MP primers was also carried out by looking at the visualization results on the UV-transilluminator, with a wavelength of 280 nm. PCR amplification in male *C. sulphurea* birds was also observed to produce one DNA band of 392 bp. However, female *C. sulphurea* produced two DNA bands of 297 bp and 392 bp, respectively. This was due to the fact that birds have different sex chromosomes, compared to mammals. The heterogametic and homogametic characters of sex chromosomes in birds were discovered in females and males, as ZW and ZZ genes, respectively (Ellegren 1996). The CHD-1 gene also showed differences in Z and W alleles in female birds, due to the linkage between their positions (Griffiths & Korn 1997).

According to Garofalo et al. (2016), NP were forward primers that attached to both the CHD-1W and CHD-1Z on the W and Z sex chromosomes, respectively. There were also two reverse primers, namely P2 and MP, which anneal to CHD-1Z and CHD-1W genes on the Z and W sex chromosomes, respectively. Therefore, in female birds, two DNA bands (Z and W) of 297 and 392 bp were observed to have occurred, compared to the males (Z) only in size of 392 bp.

Furthermore, the visualization under UV-transilluminator compared to Hyperladder 100 bp DNA Marker showed PCR products with length around 297 bp and 392 bp. The electrophoresis of the amplified CHD-1 gene was also presented in Figure 2. Samples coded SD1, SD2, SD3, SD4, and SB1 were observed to have produced clear DNA bands, compared to the very thin display of SB2, SB3 and SB4. These thin observations made visualization very difficult to conduct. Also, the 1.5% agarose gel electrophoresis showed that the blood samples produced a clear DNA band, compared to the thin display of the plucked feathers. According to Harvey et al. (2006), the amount of DNA that were isolated from a feather sample was not as much as those from the blood. However, the DNA produced from plucked feather samples was easier to degrade.

The electrophoresis of the amplified CHD-1 gene (*C. sulphurea* blood samples) compared with control male and female *Cacatua sp.,* were also presented in Figure 3. This comparison showed the PCR amplification results of the CHD-1 gene on SD1, SD2, SD3, SD4, with the production of one DNA band at 392 bp. This was in line with the results from a research on curve-billed birds by Purwaningrum et al. (2019), which discovered that male birds produced one DNA band of 392 bp, as a result of CHD-1 gene amplification on the Z sex chromosome, with the females producing two bands of 392 bp from the process. Lee et al. (2008) also stated that amplification of the CHD-1 gene segment in male birds only produced one fragment of DNA amplicon from the Z chromosome, with two fragments displayed in females from the Z and W alleles.

Based on the results of electrophoresis in Figure 3, sex determination using the PCR method on the small yellow-crested cockatoo (*C. sulphurea*), was interpreted as shown in Table 4. This interpretation indicated that the four *C. sulphurea* birds tested were male, since only single DNA band of 392 bp was produced in all samples. This interpretation was also in line with the previous studies conducted by Lee et al. (2008) and Purwaningrum et al. (2019) on the curved-billed birds, as well as by Savitri et al. (2021) that had successfully conducted the molecular bird sexing on Sulphur-crested Cockatoo (*Cacatua galerita*) and Hidayat et al. (2021) on Tanimbar Cockatoo (*Cacatua goffiniana*) by PCR amplification.

Table 4. Interpretation of the results of sex determination of *C. sulphurea* birds by PCR method.

No	Sample Code	Electrophoresis Results	Interpretation
1.	**SD1**	single DNA band	Male
2.	**SD2**	single DNA band	Male
3.	**SD3**	single DNA band	Male
4.	**SD4**	single DNA band	Male
5.	**SB1**	Single DNA band	Male
6.	**SB2**	Unclear	Unidentified
7.	**SB3**	Unclear	Unidentified
8.	**SB4**	Unclear	Unidentified

Based on the DNA quality for molecular bird sexing in *C. sulphurea,* samples derived from peripheral blood have a better quality than those from plucked feathers, which were used as a source of DNA. In male *C. sulphurea*, amplification of the CHD-1 gene generated a single DNA fragment in size of 392 bp for the Z chromosome, through the use of NP and P2 primers. However, female birds generated double DNA fragments, in sizes of 297 bp and 392 bp in the W and Z chromosomes, respectively.

In Molecular bird sexing of *C. sulphurea*, the extracted DNA from peripheral blood samples have a better quality when compared to samples from plucked feathers. Amplification of the CHD-1 gene in male *C. sulphurea* generated only a single DNA fragment in size of 392 bp, so the four of tested *C. sulphurea* in this study were male birds. Although extracted DNA template from peripheral blood samples have a better quality, we suggested that to sex determine of *C. sulphurea* which is an endangered bird to use an extracted DNA from non-invasive samples as plucked feather, because it was easier to perform, low risk, more convenient and has a low stress level for birds (Ratri 2020).

AUTHORS CONTRIBUTION

INDR carried out all of laboratory works, data analysed and wrote the draft manuscript, IP took care of the research permit and collected the field samples, WPN collected and handled the field samples, AH designed the research, supervised all of the research process and finished the draft manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no competing interest

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