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Short Communications

Using Feathers for Molecular Sexing of Straw-headed Bulbul (*Pycnonotus zeylanicus*) Offsprings

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ABSTRACT
Sex determination of of straw-headed bulbul offspring was carried out from 27 offspring's plucked feather samples in a captive breeding program. Using direct PCR, this study provided more evidences that feather samples are reliable as a source of DNA for non-invasive and effective molecular sexing. The study also revealed that the offspring sex ratio of straw-headed bulbul was slightly inclined towards males, but there was no significant difference from the value of 0.5.

Straw-headed bulbul (*Pycnonotus zeylanicus*) was a common bird and widespread from Myanmar, Thailand, Malaysia, Singapore to Sunda Island (BirdLife International 2001), but recently it is rarely found throughout its range, and it seems to be extinct in Southern Myanmar, Southern Thailand, and Java (Atlas Burung Indonesia 2020; Fishpool et al. 2020). The bird is one of the most popular and expensive song pet birds. For that reason, it is mostly trapped and rapidly decreased in numbers (Bergin et al. 2018). In 2018 the species was up-listed as *Critically Endangered Species* in IUCN Red List (BirdLife International 2018).

The breeding program of the straw-headed bulbul has been developed in Indonesia for around 20 years, mainly for commercial purposes (Lestari et al. 2015). One of the most important components of the captive breeding program is the precise assignment of sex to make sure the paired birds are sexually different. Because straw-headed bulbul is a monomorphic bird, which means that male and female birds are difficult to be identified from their external morphological characters (Fishpool et al. 2020).

Various methods have been applied for sex determination of birds, such as behaviour observation, morphometric measurement, cloacal examination, the ratio assay between estrogen and androgen in faces, laparotomy and laparoscopy (Richner 1989; Redelman et al. 1997). However, those techniques are time consuming, invasive, and risky for the birds. Consequently, such techniques for endangered species. Alternatively, non-invasive molecular sexing has been developed (Quintana et al. 2008).

Sex determination in birds mostly applies the standard method based on the amplification by PCR of the chromo-helicase-DNA binding (CHD) genes on the W and Z chromosomes (Fridolfsson & Ellegren 1999). The PCR employs two primers which anneal to conserved exonic regions and amplify across an intron in both CHD-W and CHD-Z. Each length of the intron usually differs between the genes. As a result, the PCR products vary in terms of size. Female birds are heterogametic (ZW), while the males are homogametic (ZZ). Therefore, the amplified products should migrate in electrophoresis as two bands in females and a single band in males.

A large range of primer sets have been developed for molecular sexing (Lee et al. 2010). There are three most commonly used primer pairs for molecular sexing for bird; they are the *CHD1*-linked primers P2/P8 (Griffiths et al. 1998), 1237L/1272H (Kahn et al. 1998), and 2550F/2718R (Fridolfsson & Ellegren 1999). These primer sets have been applied for molecular sexing for Indonesian bird species (Yuda 2008; Wirastika et al. 2015).

The genetic material for bird sexing mostly is blood samples, which consists of nucleated erythrocytes and are rich of nuclear DNA. However, blood sampling is considered invasively and logistically difficult (Horváth et al. 2005). In addition, for endangered species, it can be difficult to obtain research permits for more intrusive sampling methods. As an alternative, biological samples such as feather, buccal cells, faecal matter and post-hatched egg-shell membrane can be collected in the field with minimal disturbance to the species's study (Saputra & Yuda 2020; Wirastika et al. 2015; Yuda & Saputra 2021; Yuda et al. 2020; Ushine et al. 2016).

The type of sample has an effect on the likelihood of effective DNA extraction. For example, large primary, secondary, and tail feathers are preferable for genetic material samples compared to smaller plumulaceous feathers (Vili et al. 2013). Unfortunately, the available samples for this study were down feathers of straw-headed bulbul offspring. Since the number of feather available was limited, direct PCR was applied for molecular sexing of the bird. Direct PCR (dPCR) is a method of DNA amplification that enable PCR amplification without any prior DNA purification from samples due to the enzyme's resistance to inhibitors present in sample components. Comparison among six direct PCR-type DNA polymerases are commercially available, Miura et al (2013) found that KOD DNA polymerases is the most resistant to inhibitory blood components and/or detergent. The KOD also performed well when it applied to molecular sexing of water bird using blood as templates in direct PCR (Pratomo et al. 2021). For that reason, it was assumed that KOD DNA polymerase would also work well on direct PCR using down feather. The objective of this study is to assess the effectiveness of using down feather for direct PCR to identify the sex of straw-headed bulbul's offspring and to measure the sex ratio of hatched offspring in captivity.

Five plugged down feathers per bird were collected from 2-3 weeks nestling of straw-headed bulbul, provided by a commercial bird breeder in Yogyakarta, Indonesia, from September 2017 to February 2018. The feathers

were stored in separated envelope for each bird to minimize the contamination among samples and were kept in freezer (-20 ºC). For direct PCR template, one down feather was cut around 2 mm on the tip calamus using new sterilized razor blade for each sample. The PCR used the primer set of 2561-w (TAC GAG AAC GTG GCA ACA GAG) and 2728-w (CCA GTG CTT GTT TCC TCA ATT C) to amplify CHD-W and CHD-Z genes, with fragment lengths for about 400 and 650 bp respectively. The PCR mix, in a volume of 10 µL, contained DNA template, 0.2 mM each dNTP, 1x PCR Buffer, 1.5 mM $MgCl_2$, 1 U KOD FX Plus Neo DNA polymerase (Toyobo Co, Ltd), 0.3 µM each primer (2561-w /2728-w). The direct PCR was performed on the following cycle conditions: 94ºC for 2 minutes; 40 cycles of 98ºC for 10 seconds, 56ºC for 30 seconds, 68ºC for 30 seconds; and final extension at 68ºC for 7 minutes. PCR products were separated by running on 2% agarose gel and visualized under UV light in KODAK Gel Logic 2200 Imaging System.

The sex ratio was measured based on total samples and per egg clutch. The nestling (29 individuals of 18 clutches) used in this study was the offspring fr five pairs. Total sex ratio measures the proportion of males and females. Meanwhile, sex ratio per clutch was counted as the average of sex ration of the clutches that all eggs in the clutch was hatched. Only in 9 out of 18 clutches that all eggs laid were hatched.

All samples from the plucked down feather of straw-headed bulbul were successfully amplified using direct PCR. Eleven samples showed double band, indicating a female offspring, and the other sixteen samples with single band (males). The sizes of the bands were about 650 bp and 400 bp for the female, and was about 650 bp for the male (Figure 1). The length of CHD1-W gene of straw-headed bulbul was shorter than its CHD-1-Z gene. This result was in accordance with previous studies which using the primer set of 2550F/2718R on different bird species in Indonesia, such as Bali starling (*Leucopsar rothchildi*) (Wirastika et al. 2015), Maleo (*Macrocephalon maleo*) (Yuda

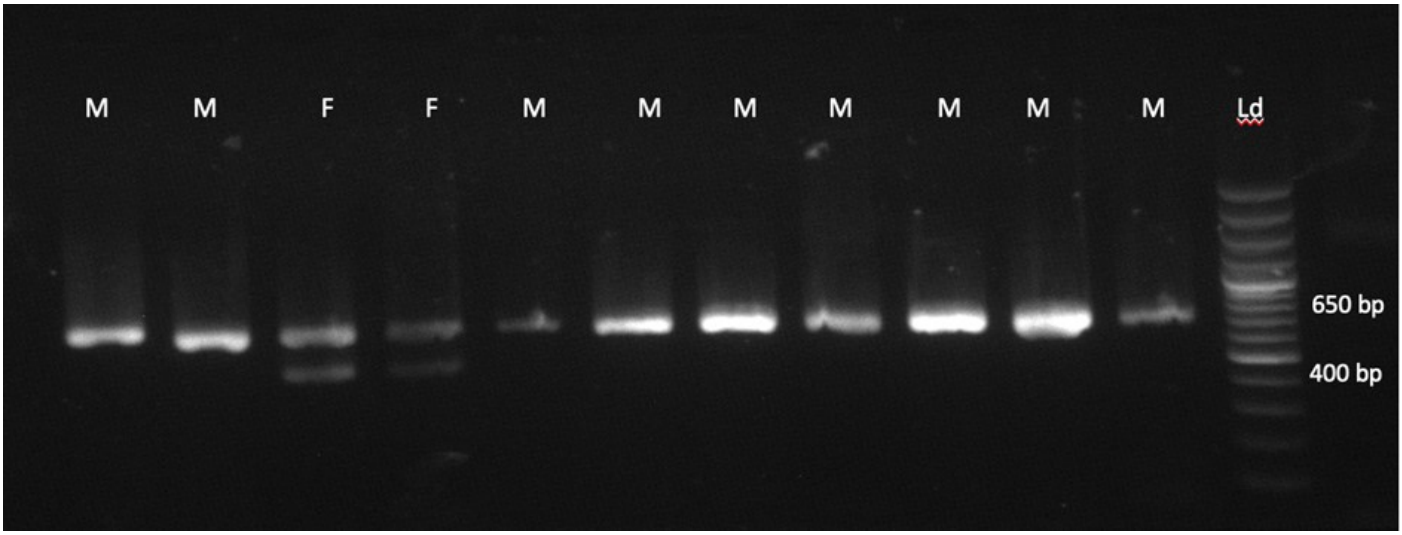

Figure 1. The PCR products of the CHD-1 W and CHD-1 Z of straw-headed bulbul (M-male; F –female; Ld – molecular ladder).

& Saputra 2021), 9 bird of prey species (Yuda et al. 2020), and 56 birds species of 13 families (Sulandari & Zein 2012). In contrary, using P2/P8 primer set for molecular sexing for two different species of bulbul (*Pycnonotus* spp), Pamulang & Hanyarto (2021) reported that the length of CHD1-W gene (400 bp) was longer than CHD1-Z (300 bp). The same result was also found in Tanimbar Cockatoo (*Cacatua. goffiniana*) (Hidayat et al. 2021).

Compared to blood samples, feathers provide lower DNA yields. For that reason, using blood as DNA material was preferable and have provided a better result on molecular bird sexing (Hidayat et al. 2021; Pamulang & Haryanto 2021). However, feather sampling is easier, faster, and less invasive. The handling time of the bird is reduced so that it may reduce stress on the captured birds. In addition, feathers have other values such as for isotopes analysis and trace elements as well as age determination. Feather samplings have been applied as alternative DNA sources at migration monitoring stations (Smith et al. 2003). The results of this study supports the previous studies that the plucked feather is a reliable DNA source for sexing wild birds (Harvey et al. 2006; Costantini et al. 2008). Another study which was congruence with this finding was that a single plucked feather is reliable as a source of DNA, not only for sexing but also for other genetic studies (Segelbacher 2002). In addition, the use of direct PCR makes it possible for more rapid determination of sex, as the reaction without isolation or purification of DNA reduces the analysis time.

Most bird captive breeders believe that the sex of the nestling from the same clutch is always male and female. On the other hand, this study revealed that only 5 of 9 complete hatched clutches have both sexes for two nestlings in the same clutch (Table 1). The sex ratio of all nestling is slightly biased to male (60:40), but there is no significant difference from 0.5 ($p=0.33$). Meanwhile, the offspring sex ratio for the complete hatched offspring in the same clutch were 11 males and 7 females, and there was no significant difference from 0.5 ($p=0.34$).

This finding revealed that in captive breeding the offspring sex ratio of straw-headed bulbul is a typical sex ratio to most birds (0.5). Based on the estimation of the sex ratio of 140 offspring from 114 species, mostly they were 0.5, and only 11 inclined towards males (Donald 2007).

This study provided more evidences that a plucked feather sample is reliable as a source of DNA for molecular sexing. The use of the down feathers of straw-headed bulbul as DNA template on direct PCR requires less time and it is non-invasive, as this may apply for other endangered species. The study also revealed that the offspring sex ratio of straw-headed bulbul was slightly inclined towards males, but there was no significant difference from 0.5 value.

Table 1. The sex of straw-headed chicks in the captive breeding program based on molecular sexing.

Parent	Clutch	Number of eggs laid	Number of hatched eggs	Sex	
				Male	Female
I	1	2	1	-	1
	2	2	2	1	1
	3	2	1	-	1
	4	2	1	-	1
II	1	2	1	1	-
	2	2	2	2	-
	3	2	2	-	2
III	1	2	1	1	-
	2	2	1	1	-
	3	2	2	1	1
IV	1	2	2	1	1
	2	2	2	1	1
	3	2	2	2	-
	4	2	2	1	1
	5	2	2	2	-
	6	2	2	-	2
	7	2	2	2	-
V	1	2	1	1	-
Total			29	17	12

AUTHORS CONTRIBUTION

PY designed and carried out the laboratory works and wrote the manuscript. WW designed the research. All authors contributed to this research and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no competing interests.

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