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Use of swab for DNA sampling from confiscated raptors for molecular sexing

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Abstract. The objective of the study was to evaluate the feasibility and efficiency of using swab to collect tracheal and cloacal epithelial cells of confiscated raptor bird for genetic studies. Commercial swab kits were used to collect samples from 34 individuals of 10 raptor species, and as comparison blood samples from the same individual were also analyzed. FavorPrep^{TM} Blood Genomic DNA Extraction Mini Kit dan FavorPrep^{TM} Tissue Genomic DNA Extraction Mini Kit were used respectively to extract DNA from blood and epithelial cells. All DNA extracted from blood were successfully amplified for assignment of sex. On the other hand, the DNA extracted from buccal and cloacal swabs were only respectively 71% and 9% successfully amplified. This result suggests the potential used of buccal swabs for genetics studies of raptor, with further optimization for a better result.

1. Introduction

All raptor birds (Family Accipitridae, Pandionidae and Falconidae) are protected by law in Indonesia [1]. However, due to high demands of the raptor as pet bird, the black market and illegal trade of the raptors is still existing [2]. This threat along with other factors, such as habitat lost and pollution, have caused the raptors faced endangerment. Indonesia harbors 72 raptors species [3], among of them five species have been listed as Globally Threatened species (1 sp. *Critically Endangered*, 1 sp. *Endangered* and 3 sp. *Vulnerable*) in IUCN Red List [4].

As a result of law enforcement, recently more than 100 raptors have been confiscated and in the process of rehabilitation in different rehabilitation centers in Java. Unfortunately, the information about the confiscated birds was very limited. Even the basics information such as the origin or where the birds were catch, and their sex were unavailable. This information is crucial for deciding where the best site for releasing the birds.

Molecular techniques are now available and providing reliable tools to investigate that information, which may not be solved by conventional technique. For example, DNA analysis guided the release into the wild of Blue-and-Yellow Macaws (*Ara ararauna*, Psittaciformes, Aves) from the illegal trade [5]. To determine the sex of White-tailed Eagle (*Haliaeetus albicilla*) nestlings PCR-based amplification of chromo-helicase-DNA binding 1 (*CHD1* genes was used for DNA sexing [6].

Molecular analysis requires a good sample of genetic material. Most genetic study on birds preferred blood as source of DNA [i.e 7,8], since red blood contains nucleus DNA. However, blood sampling requires the capture of the birds and considered as invasive, which may increase stress level and might results in unusual behavior or in nest desertion. Brown and Brown reported that blood sampling reduced Cliff Swallows (*Petrochelidon pyrrhonota*) annual survival [9].

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To minimize the negative impact of blood sampling, genetic studies on birds have utilized other genetic materials. Molted feathers were used for genetic study of water bird [10] and for species identification [11]. Other DNA sources for genetic study of birds included buccal swab [12,13], eggshell membrane [14], and even feces [15,16].

Here we are comparing three different DNA sampling techniques as a template for PCR, to determine the sex of confiscated raptors in Indonesia. Knowing the sex of birds prior releasing in the wild is important to optimize the success of the program in accordance with the existing demographic of resident birds.

2. Methods

A total of 34 individuals, consisted of nine raptor species (Table 1) were sampled from two rehabilitation centers: Flora and Fauna Station (SFF, Yogyakarta; n=13,) and Cikananga Wildlife Center (West Java; n=21,). Each bird was sampled its blood, tracheal swab, and cloacal swab. All sampling was carried out by the Veterinary of the Centers. Each swab sample was stored in separated microtubes, meanwhile the blood samples were kept in dry filter papers and store in separate envelope. All samples were stored in freezer until extraction.

Table 1. Raptor species used in the study

	Species	Scientific name	Sample size (n)
1,	Changeable Hawk-eagle	*Nisaetus cirrhatus*	19
2.	Crested Serpent Eagle	*Spilornis cheela*	8
3.	Javan Hawk-eagle	*Nisaetus bartelsi*	1
4.	White-bellied Fish Eagle	*Ichthyophaga leucogaster*	1
5.	Grey-headed Fish Eagle	*Ichthyophaga ichtyaetus*	1
6.	Black Eagle	*Ichtinaetus malaiensis*	1
7.	Black Kite	*Milvus migrands*	1
8.	Besra	*Tachyspiza virgata*	1
9.	Sunda Honey Buzzard	*Pernis ptilorhyncus*	1

We used spin column DNA extraction kits (DNeasy Blood & Tissue Kit, gSYNC DNA Extraction Kit & FavorPrep); following the provided protocol by the company. To determine the sex of the bird, we used PCR based amplification of CHD1 genes, using the universal primer set for sexing: *2550F* (5'-GTTACTGATTCGTCTACGAGA -3') and *2718R* (5'-ATTGAAATGATCCAGTGCTTG -3') [17]. The PCR was run in *Applied BioSystems Veriti* thermocycler, on 10 uL reaction, which consisted of 10-20 ng DNA; 2x PCR Buffer for KOD Neo; 5 pmol each primer; 0.15 mM each dNTP & 1 U KOD DNA polymerase. Each amplification was run with three different DNA samples, respectively extracted from blood, tracheal and cloacal swab. The cycles condition was as follow 1 cycle at 94℃ for 2 min followed by 35 cycles of 98℃ for 10 sec, 53℃ for 30 sec and 68℃ for 45 sec; and final extension at 68℃ for 7 minutes. The amplicon was stained with ethidium bromide and separated in 2% agarose gel (in 1x TAE buffer), 100v for 30 minutes. We then visualized the gel in the *Kodak Gel Doc System*. Male bird is expected to show single band (Z-Z), meanwhile female bird shows double bands (W-Z).

3. Results and Discussion

All DNA extracted from blood were successfully amplified for assignment of sex (Figure 1). Female birds showed two bands, indicated CHD1-W and CHD1-Z genes. Meanwhile male birds showed only one band, indicated double CHD1-Z. The size of CHD1-Z (650-670 bp) is larger than CHD1-w (435-450 bp). This size differences between CHD1-Z and CHD1-W of the raptors assessed in this study size agrees with other studies using the same primer set [17,18].

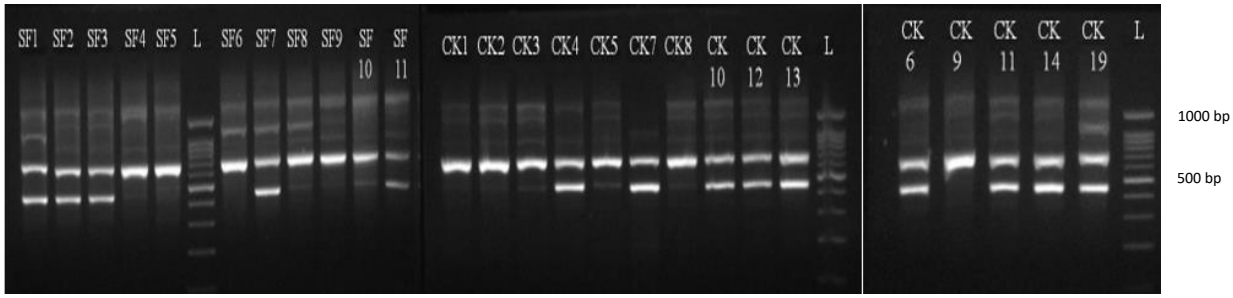

Figure 1. The PCR based amplification of CHD1 genes of confiscated raptors using primer set of 2550F/2718R What is CK, SF?

On the other hand, the DNA sexing using swab, both tracheal and cloacal, as DNA template was not successful as using the DNA from blood. The DNA which was extracted from tracheal and cloacal swabs were less successful for amplification, respectively only 85% and 45% successfully amplified (Figure 2; Table 2). The failure of amplification may corelated with the little amount of or DNA extracted from the two swabs. Other possibility is that the PCR condition was not optimum for small amount of DNA template. However, further PCR optimization may necessary to prove this hypothesis.

For samples which were all amplified for all type of DNA template (15 samples), the sex determination was all consistent (Table 1). Only one sample (CK20) showed inconsistent result. Using DNA from blood as template indicate female (2 bands), meanwhile using DNA from tracheal swab indicated male bird (1 band). Most likely that allelic dropout was occurred in the sample for the later. Previous studies suggested that low DNA quantity may lead to allelic dropout or false allele [19–21]. Even though further optimization on DNA extraction as well as on PCR is still needed. This study reveals the potential used of less invasive sampling technique (swab) for genetic study of bird.

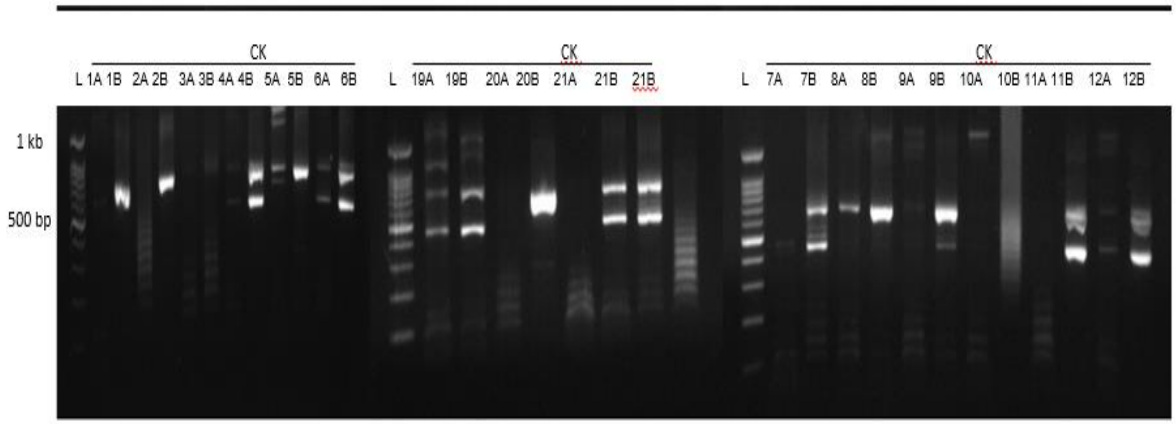

Figure 2. The PCR based amplification of *CHD1* genes of confiscated raptors using universal primer set of 2550F/2718R.
(Note: CK indicates samples collected from Cikananga, A and B after the sample numbers indicates sampling techniques, respectively for cloacal (A) and tracheal (B) swabs; L-ladder)

Table 2. The sex of confiscated raptor as result of PCR-based assessment using different DNA templates

	Sampel Code	Species	Sample type*		
			B	CS	TS
1.	CK 1 (Jajat)	Changeable Hawk-eagle - Dark Morph	♂	x	♂
2.	CK 2 (Thor)	Changeable Hawk-eagle - Light Morph	♂	x	♂
3.	CK 3 (Floyd)	Changeable Hawk-eagle - Light Morph	♂	x	♂
4.	CK 4 (Darwin)	Crested Serpent-eagle	♀	x	x
5.	CK 5 (Jona)	Changeable Hawk-eagle - Light Morph	♂	♂	♂
6.	CK 6 (Maxi)	Crested Serpent-eagle	♀	♀	♀
7.	CK 7 (Weda)	Changeable Hawk-eagle - Intermediate Morph	♀	x	♀
8.	CK 8 (Lewis)	Changeable Hawk-eagle - Light Morph	♂	♂	♂
9.	CK 9 (Bulbul)	Javan Hawk-eagle	♂	x	♂
10.	CK 10 (Zae)	Changeable Hawk-eagle - Light Morph	♀	x	♀
11.	CK 11 (Daan)	Changeable Hawk-eagle - Dark Morph	♂	x	♂
12.	CK 12 (Budi)	Changeable Hawk-eagle - Dark Morph	♂	♂	♂
13.	CK 13 (Siva)	Changeable Hawk-eagle - Dark Morph	♂	x	♂
14.	CK 14 (Kim)	Changeable Hawk-eagle - Dark Morph	♂	♂	♂
15.	CK 15 (Cool)	Crested Serpent-eagle	♂	x	♂
16.	CK 16 (Acong)	Changeable Hawk-eagle - Light Morph	♀	x	♀
17.	CK 17 (Mini)	Crested Serpent-eagle	♂	♂	♂
18.	CK 18 (Mulan)	Changeable Hawk-eagle - Light Morph	♂	x	♂
19.	CK 19 (Dego)	White-bellied Sea Eagle	♀	♀	♀
20.	CK 20 (Selvi)	Changeable Hawk Eagle - Light Morph	♀	x	♂
21.	CK 21 (Bande)	Changeable Hawk Eagle - Light Morph	♂	x	♂
22.	SF1(KPB1)	Grey-headed Fish Eagle	♀	x	♀
23.	SF2 (KPB2)	Changeable Hawk-eagle - Light Morph	♀	♀	♀
24.	SF3 (KPB3)	Changeable Hawk-eagle - Light Morph	♀	x	x
25.	SF4 (KPB4)	Crested Serpent Eagle	♂	x	x
26.	SF5 (KPB5)	Black Eagle	♂	x	x
27.	SF6 (KPB6)	Black Kite	♂	♂	♂
28.	SF7 (KPB7A)	Crested Serpent Eagle	♀	♀	♀
29.	SF8 (KPB7B)	Crested Serpent Eagle	♂	x	x
30.	SF9 (KPB8)	Crested Serpent Eagle	♂	♂	♂
31.	SF10 (KPB9)	Besra	♂	♂	♂
32.	SF11(KPB10)	Changeable Hawk-eagle - Intermediate Morph	♀	♀	♀
33.	SF12 (KPB11)	Changeable Hawk-eagle - Light Morph	♂	♂	♂
34.	SF13 (KPB12)	Sunda Honey Buzzard	♀	♀	♀

*Note: * - extracted DNA from B (blood), CS (cloacal swab), TS (tracheal swab); x- unidentified (the samples were not amplified in PCR)*

4. Conclusion

This study revealed that DNA extracted from buccal and cloacal swabs were not as good as DNA extracted from blood for assignment of sex of the raptor. Its amplification rates were only respectively

71% and 9%. Further optimization for a better result is needed to apply this less invasive DNA sampling for further genetics studies.

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