Sources of DNA and amplification of COI gene of Changeable Hawk-eagle *Nisaetus cirrhatus* (J.F. Gmelin 1788)

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Abstract. The mitochondrial gene of cytochrome c oxidase I (COI) has been commonly used in population genetics, systematics, phylogeography as well as DNA barcoding of many animal species including birds. This study aimed to evaluate the sources of DNA and condition of PCR reaction for amplification of COI gene of changeable hawk-eagle (*Nisaetus cirrhatus* Gmelin, JF, 1788). DNA was extracted from single fresh plucked feather and from cloacal swab of five individuals. The DNA extraction was conducted using Geneaid™ DNA Isolation Kit (Tissue). Amplification of COI gene was done in a reaction mixture containing 1x PCR buffer, 0.2mM dNTP, 2mM to 3mM MgCl₂, 1µM of each forward and reverse primers, 1µl to 3.5 µl DNA template, 1U taq polymerase and H₂O to reach a total volume of 20µl. The PCR cycles were 95°C for 1 minute followed by 5 cycles of 95°C for 1 minute, 45°C for 1.5 minutes, and 72°C for 1.5 minutes. This was followed by 30 cycles of 1 minute at 95°C, 1.5 minutes at 50°C, and 1.5 minute at 72°C. One cycle of a final extension was done for 5 minutes at 72°C. The amplification products were visualised using agarose gel electrophoresis. The results showed that DNA extracted from feather had higher concentration than from cloacal swab. Moreover, DNA extracted from plucked feather demonstrated better amplification product with the fragment size of 750bp. The volume of DNA template from plucked feather that resulted in reproducible amplification product was 3.5 µl and the MgCl₂ concentration was 3mM.

1. Introduction

Changeable Hawk-eagle or Crested Hawk-eagle (*Nisaetus cirrhatus* Gmelin, JF, 1788) is a bird of prey or raptor that belongs to the family of Accipitridae. This species has wide distribution in Indian subcontinent and southeast Asia [1]. In Indonesia, Changeable Hawk-eagle is found in Greater Sunda including Sumatra, Java and Bali [2]. There are five subspecies of Changeable Hawk-eagle ie. N. c. cirrhatus (J. F. Gmelin, 1788), N. c. ceylanensis (J. F. Gmelin, 1788), N. c. andamanensis (Tytler, 1865), N. c. limnaeetus (Horsfield, 1821) and N. c. vanheurni (Junge, 1936) [1]. However, in the field, three types of Changeable Hawk-eagle are known. They are Changeable Hawk-eagle dark morph eagle, Changeable Hawk-eagle light morph and Changeable Hawk-eagle intermediate or pale morph [3]. The identification is often hard because their colour of plumage from bird to bird can vary greatly.

The identification of Changeable Hawk-eagle is commonly done by observing and comparing the bird habits, size, shape and pattern of plumage [4]. Identification can also be done using voice. Technological developments led to the development of various DNA-based analysis techniques that
can be used to identify and detect genetic diversity of a species. Molecular markers have been used to analyse phylogeny, identification and genetic diversity of Changeable Hawk-eagle. The most commonly used DNA marker is mitochondrial COI gene [5]. Studies on genetic diversity of Changeable Hawk-eagle in Indonesia are not widely reported. A study was available on genetic diversity of different morphs of Changeable Hawk-eagle that were confiscated by Natural Resources Conservation Agency (BKSDA) Kulon Progo, Yogyakarta, Indonesia [6]. The study found that the genetic distance between different morphs was 0.015, while the genetic distance among same morphs was 0.001 [6]. Other studies from different areas in Indonesia need to be conducted.

Cytochrome c oxidase I (COI) gene is used in population genetics, systematics and phylogeography. The diversity of COI region, make it ideal for identification. The COI gene has also been employed in DNA barcoding of animal species. In Indonesia, the COI gene has been used for DNA barcoding of members of the family Accipitridae including Nisaetus bartelsi, Nisaetus cirrhatus, Haliaeetus leucogaster, Spilornis cheela, and Haliastur indus [7]. Successful amplification of COI gene requires optimal condition of all amplification reagents including DNA template as well as optimal PCR cycles. Therefore, it is necessary to determine the tissues used as DNA sources.

DNA of bird can be extracted from blood, cloacal swab, buccal swab and faeces [8]. Taking blood sample is an invasive sampling method which can cause animal stress, while both cloacal swab and buccal swab are non-invasive methods. Sampling techniques that are non- or less-invasive are important because it will minimize stress to organisms [9]. In avian genetic, other good source of DNA is feather. DNA from feather has been used in genetic structure and phylogeography studies [10, 11].

The aim of this research was to evaluate feather and cloacal swab as DNA sources from changeable hawk-eagle. This will provide alternatives of DNA source beside the used of blood sample. The other aim was to evaluate the condition of PCR reaction for amplification of COI gene of changeable hawk-eagle. When the optimal condition is obtained, the condition can be used in the future for further analyses such as molecular identification and genetic diversity analyses.

2. Methods
2.1. Sample collection
Two types of samples were used in this study, newly plucked feathers and cloacal swab. Samples were taken from five individuals of Changeable Hawk-eagles at Bali Bird Park, Gianyar, Bali, Indonesia. The five individuals are in a healthy condition. The feathers were plucked from the abdomen area and then were kept in separate envelopes. We found that it was easier to pluck feathers from abdomen area and it was not hard to pull the feather out. Three feathers were taken from each individual. Cloacal swab was done by swabbing cloaca gently several times using cotton bud. The cotton buds were then place in a tube containing 95% ethanol. All sampling procedures were conducted by the help from veterinarian and staff of Bali Bird Park.

2.2. DNA extraction
DNA extraction was conducted using Geneaid™ DNA Isolation Kit (Tissue) following manufacturer instruction with modification. Single feather for each individual was used as sample. The feathers were soak in 3% bleach for 30 seconds and rinse quickly with sterile water [12]. The calamus of the feather (Figure 1.) was cut using sterile scissor and sliced further into fine pieces and put into a mortar then was ground using pestle to pulp. Single calamus was used for each individual. As much as 200µl of GT buffer was added, homogenized and transfer into microtube. Proteinase K (20µl) was added and the mixture was incubated for 1 hour at 60ºC. After that, 200µl of GBT buffer was added and incubated for 20 minutes at 60ºC.

The mixture was centrifuged at 14000 rpm for 2 minutes then the supernatant was transfer to a new microtube. RNase A (4µl) was added and incubated at room temperature for 5 minutes. Absolute ethanol (200µl) was added and shook for 10 seconds. The mixture was transferred to the GS Column
and centrifuged at 14000 rpm for 2 minutes. The GS Column was then put to a new 2 ml Collection Tube.

As much as 400μl of W1 Buffer was added to the GS Column then centrifuged at 14000 rpm for 30 seconds. The flow-through was discarded and then GS Column was placed in the 2ml Collection Tube. Wash buffer (600μl) was added to the GS Column and centrifuged at 14000 rpm for 30 seconds. After that, the GS Column was placed back in the 2 ml Collection Tube and centrifuged for 3 minutes at 14000 rpm. The dried GS Column was transferred to a new microtube and 50μl of pre-heated Elution Buffer was added to the centre of the column. After 5 minutes, the tube was centrifuged for 30 seconds at 14000 rpm.

**Figure 1.** Part of a feather (calamus) of changeable hawk-eagle used as DNA source

For cloacal swab, the tube containing cotton bud in the 95% ethanol was vortexed thoroughly. Then the cotton bud was removed and the tube was centrifuged for 10 min at 14000 rpm. The ethanol was discarded and the pellet was air dried. After that, the DNA was extracted with the same method as DNA extraction from feather above.

DNA concentration was measured by measuring absorbance at λ260 nm using nanodrop (Simplino, Biochrom). Absorbance at λ260=1 indicated that the DNA concentration is 50 ng/μl [13]. The ratio of absorbance at λ260 nm λ280 nm was calculated to determine the purity of DNA.

### 2.3. Amplification of COI gene

The COI gene of changeable hawk-eagle was amplified using primer BirdF1-TTCTCCAACCACAAGACATTGGCAC and BirdR1-ACGTGGGAGATATTTCCAAATCCTG [5] in a thermal cycler (Bior). The total volume of reaction mixture was 20 μl containing 1x PCR buffer (Promega), 0.2mM dNTP (Promega), 2mM to 3mM MgCl$_2$, 1 μM of each forward and reverse primers, DNA template (ranged from 1μl, 2μl and 3.5μl), 1 U Taq polymerase (Promega) and H$_2$O. The PCR cycles were 1 cycle of 95°C for 1 minute followed by 5 cycles of 95°C for 1 minute, 45°C for 1.5 minutes, and 72°C for 1.5 minutes. This was followed by 30 cycles of 1 minute at 95°C, 1.5 minutes at 50°C, and 1.5 minutes at 72°C. One cycle of a final extension was done for 5 minutes at 72°C [5].

### 2.4. Visualization of PCR products

PCR products were visualized using 1.2% agarose gel in TAE buffer. As much as 5μl of PCR product was loaded into the gel. As size marker, 5μl of 100 bp ladder (Promega) was included in the gel. Electrophoresis was done at 100 V for 45 minutes. The gel was then stained using ethidium bromide and PCR products were observed using UV transilluminator.

### 3. Results and Discussion

The average concentration of DNA extracted from single newly-plucked feather was 3.76ng/μl, while from cloacal swab was 2.74ng/μl. Table 1 shows the comparison of DNA concentration and DNA quality from the two difference sources.
The ratio of absorbance at $\lambda 260$ nm $\lambda 280$ nm ($A_{260}/A_{280}$) ranged from 1.651 to 2.041 at DNA extracted from feather, while for DNA extracted from cloacal swab the range was 1.375 to 2.695. The DNA is considered pure when the ratio of $A_{260}/A_{280}$ is 1.8 to 2.0 [14]. For DNA extracted from feather, only sample no. 1 had $A_{260}/A_{280} < 1.8$ while for DNA from cloacal swab, sample no. 2 and 3 had $A_{260}/A_{280} < 1.8$ and sample no. 5 had $A_{260}/A_{280} > 2.0$.

In amplification of $COI$ gene, three volumes of DNA template were tested. The DNA concentrations were 1µl, 2µl and 3.5µl. The concentrations of MgCl$_2$ tested were 2mM and 3mM. When 1µl DNA template was used, and the concentration of MgCl$_2$ was 2mM, no PCR product was obtained both for DNA from feather and from cloacal swab. The volume of DNA template was increased to 2µl with 3mM MgCl$_2$. PCR product was observed from sample no. 3 of DNA extracted from feather, while the PCR failed to produce PCR product for DNA extracted from cloacal swab, (Figure 2).

| Source  | No | DNA concentration (ng/µl) | A260/A280 | Source  | No | DNA concentration (ng/µl) | A260/A280 |
|---------|----|--------------------------|-----------|---------|----|--------------------------|-----------|
| Feather | 1  | 3.7                      | 1.651     | Cloacal swab | 1  | 1.4                      | 2.043     |
|         | 2  | 4.0                      | 1.844     |         | 2  | 2.0                      | 1.339     |
|         | 3  | 4.5                      | 1.888     |         | 3  | 2.2                      | 1.375     |
|         | 4  | 2.8                      | 1.990     |         | 4  | 4.5                      | 2.010     |
|         | 5  | 3.8                      | 2.041     |         | 5  | 3.6                      | 2.695     |
| Average |    | 3.76                     |           | Average |    | 2.74                     |           |

**Figure 2.** Amplification of $COI$ gene of changeable hawk-eagle using 2 µl of DNA extracted from feather (a) and from cloacal swab (b). Number 1 to 5 indicates the individuals of changeable hawk-eagle used as samples. Red arrow indicates PCR product

DNA concentration was increased further to 3.5µl and concentration of MgCl$_2$ used was 3mM. The $COI$ gene amplification was successful for all DNA sample extracted from feather (Figure 3a). The size of PCR product was 750 bp. This DNA fragment size is similar with previously reported study of $COI$ gene in bird using BirdF1 and BirdR1 primer pair [5]. Sample no. 3, resulted in the brightest band indicated many copies of amplicon produced. This may due to high concentration of DNA template in sample no. 3. The increase of MgCl$_2$ concentration to 3mM may also contributed to the successful amplification of $COI$ gene. The concentration of MgCl$_2$ is one of the important factors in PCR specificity and sensitivity. MgCl$_2$ affected the melting of double stranded DNA and affected
activity of Taq DNA polymerase [15, 16]. High concentration of MgCl₂ can increase the efficiency of the PCR amplification but, Low concentration of MgCl₂ leads to no PCR product, while too high of MgCl₂ concentration will reduce PCR specificity [15].

Amplification of COI gene using DNA template extracted from cloacal swab only produced faint band for sample no. 4 (Figure 3b). The concentration of DNA template affected the success of gene amplification. Sample no. 1, 2 and 3 had low DNA concentration, therefore the amount of DNA template for amplification was not enough. The low DNA concentration obtained indicated that low number of epithelial cells collected during cloacal swabbing. The failure of amplification using DNA from cloacal swab could also due to the presence of PCR inhibitor. DNA from cloacal swab samples can contained contaminant from faeces [8]. PCR inhibitors in faeces including dead cell, microorganisms, polysaccharides, gut flora, chlorophyll, glycolipids, heparin, haemoglobin, and urea [17, 18, 19].

Sample no. 5 from cloacal swab had quite high DNA concentration (3.6 ng/µl) which was higher than DNA sample 2 extracted from feather, but sample 5 did not produce PCR product. This may due to low quality of DNA in sample 5 from cloacal swab. DNA contaminant may present in the DNA template which inhibit PCR reaction. The ratio of A260/A280 for this sample was 2.695, while the value of the ratio of A260/A280 from pure DNA is 1.8 to 2.0 [14].

![Image](image_url)

**Figure 3.** Amplification of COI gene of changeable hawk-eagle using 3.5 µl DNA extracted from feather (a) and from cloacal swab (b). Number 1 to 5 indicated the individuals of changeable hawk-eagle used as samples. Red arrows show PCR product with DNA fragment size of 750 bp

Studies on COI barcoding of bird mostly used feather as source of DNA [11] or from blood sample [7, 20, 21]. A study was published by Asawakarn et al. [8] on comparison of dried blood spot, buccal swab, cloacal swab and faeces as DNA sources to identify avian sexes by PCR. The used of dried blood spots as DNA source was reported to show best result in avian sexing using PCR, followed by buccal swab and cloacal swab. The success rate for determination of sex from dried blood spots was 100%, while from buccal swabs, cloacal swabs and faeces were 74%, 75.47% and 29.17% respectively.

Taking blood sample for DNA extraction required specific method and skill, therefore less complicated sample collection is needed. Feather has been reported as reliable source of DNA. For example, plucked feather produced similar results with blood sample in sex determination of wild birds Black-capped Chickadees (Poecile atricapilla) using PCR [22]. Feather as source of DNA has also been used in characterisation of members of the family Accipitridae using COI gene [7]. The base part of feather (calamus) is tubular part of feather that implant in skin [23]. Calamus has cellular material both outside and inside of shaft which is used as DNA source [24]. Our study confirmed that, feather is a potential source of DNA for amplification of COI gene of changeable hawk-eagle. Better PCR products were obtained when DNA from feather was used as compared to DNA from cloacal...
swab. The use of feather as DNA source is useful especially when permission is not granted to collect blood samples [22]. In addition, sampling cloacal swab can cause animal stress.

4. Conclusion
Calamus of feather is better to be used as DNA source as compared to cloacal swab. Furthermore, to obtain strong amplicon, PCR reaction need to be optimised and the DNA need to be in high quality. Further step will be sequencing the COI fragments and analysing sequences by finding the homology using BLAST search in NCBI database.

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