Phylogenetic study of Changeable Hawk-eagle (*Nisaetus cirrhatus*) based on *cytochrome-c oxidase subunit I* (COI) gene as one of the conservation efforts in genetic diversity

Y Rifaldo¹, D A Mandasari¹ ², D N Rahmawati¹ ², R Krisdayana¹ ², F Salehah¹ ², R W Retnaningtyas¹ ² ³, Suhadi¹ and D Listyorini¹ ²

¹Department of Biology. Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, Jalan Semarang, 5 Malang, Indonesia, 65145
²Biotechnology Division, Central Laboratory of Mineral and Advanced Material, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang Jl Semarang, 5 Malang, Indonesia, 65145
³Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701 United States of America (current affiliation)

Email: listyorini.aljabari@um.ac.id; suhadi@um.ac.id

Abstract. The wide distribution and abundance of Changeable Hawk-eagle (*Nisaetus cirrhatus*) across Southeast Asia has prompted several subspecies, namely *N. c. cirrhatus*, *N. c. ceylanensis*, *N. c. andamanensis*, *N. c. limnaeetus* and *N. c. vanheurni*. Despite their declining population trend due to anthropogenic threats such as illegal wildlife trading and deforestation, this species is still categorized as least concern. Therefore, in light of determining the management unit for conservation endeavour, and to reveal their phylogenetic relationships within species, a thorough phylogenetic using COI gene as a preliminary research is urgently needed. The main objectives of this study were to obtain COI gene sequences of 5 individuals (4 intermediate phase and 1 dark phase) eagles and to construct their phylogenetic tree. The stages of this research were extraction and quantification of DNA blood cells of Changeable Hawk-eagle, amplification of DNA by applying PCR method using COI gene specific primers, electrophoresis on 0.8% agarose gel and DNA sequencing by First Base Laboratories, Malaysia. The DNA sequence analysis was carried out using a MEGAX software with Neighbour Joining (NJ) method. Based on COI gene sequence, *N. cirrhatus* forming a species complex which was indicated by a monophyletic clade with smaller branches with different bootstrap values in NJ tree. Therefore, *N. cirrhatus* is closely related to *N. phillippensis*. In addition to enrich the genetic data of *N. cirrhatus*, this paper could be one effort to understand genetic diversity among eagles in the world.

1. Introduction

The Changeable Hawk-eagle (*Nisaetus cirrhatus* Gmelin, 1788) is the most widespread Asian eagle species [1]. Due to their wide distribution combined with the diverse geographical condition of Southeast Asia, this species forms five subspecies, including *N. c. cirrhatus*, *N. c. ceylanensis*, *N. c. andamanensis*, and Indonesia hosts *N. c. limnaeetus* and *N. c. vanheurni*. Changeable Hawk-eagle is distributed around Asia started from India subcontinent, Sri Lanka, Nepal, Andaman Island to Southeast Asia through Bangladesh, Myanmar, then Indochina and Malay Peninsula through Greater
Sundas (Sumatra, Java, Borneo, Sulawesi) to Philippines [2]. In addition of the two characteristics (crested and crestless) along with dark brown colour and streaks, adult Changeable Hawk-eagles generally have a total body length of 51-82 cm, a weight of 1300-1900 grams and a wingspan of 100-160 cm [2]. Moreover, Changeable Hawk-eagle has cultural value by its eagle-characteristics which represent its significant value for cultural diversity like Balinese and Javanese people [3].

Although N. cirrhatus is not a globally threatened species (Least Concern), its population tends to decline due to habitat destruction [1]. Deforestation and illegal wildlife trade even worsen the situation [4]. Meanwhile, isolated populations also elicit inbreeding potential which can affect the loss of genetic diversity [5]. This suggests that such eagle population will continue to decline if there is no efforts to maintain the ecosystems.

Phylogenetic analysis become important as a solution to provide us with information of overall biodiversity through genetic-related aspects in order to preserve the genetic diversity [6]. Phylogenetic analysis through DNA Barcoding is one of rapid solutions to help identifying the genetic distinction of a species based on its unique molecular gene Cytochrome-c Oxidase Subunit I (COI) as the marker [7]. Currently, lack genetic data of N. cirrhatus has been reported by both GenBank and BOLD Systems, and this encouraged us to conduct this research with a view to enrich the genetic data of N. cirrhatus, using the COI gene in particular.

Previous studies of Changeable Hawk-eagle which were based on Cytochrome-b (Cyt-b) gene and Control Region (CR) gene as two sections of mitochondrial genome, presented the molecular phylogeny of the N. cirrhatus complex (formerly known as Spizaetus cirrhatus) is closely related to N. philippensis and N. lanceolatus [8]. The phylogenetic tree of the N. cirrhatus complex established in this study presents the relationships of the taxa which both had ambiguous in Cyt-b and CR trees. This study reported low genetic variability within N. cirrhatus and could also be interpreted as intraspecific variation. It showed that application of different species concepts (Phylogenetic Species Concept and Biological Species Concept) would concern the taxa of the N. cirrhatus complex with its several morphological distinct characteristics [8]. Therefore, our study could be a preliminary research of N. cirrhatus by promoting phylogenetic through COI gene in order to understand the genetic diversity among eagles in the world.

2. Methods

2.1. Collection of samples

This research was conducted from February to April 2019 at the Biotechnology Division, Central Laboratory of Mineral and Advanced Material, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang. The samples of Changeable Hawk-eagle used in this study were obtained from Cikananga Wildlife Centre in Sukabumi District (five individuals consisting of 4 intermediate eagles encoded with RR13, RR14, RR15, RR16, and 1 dark phase-eagle encoded with RR19).

2.2. DNA extraction and amplification of COI gene

The blood samples were collected from 5 individuals of Changeable Hawk-eagle and stored in 1000 µl absolute alcohol at -20°C. DNA extraction of blood samples was conducted using High Pure PCR Template Preparation Kit (Roche, 11796828001). The primers used for amplification were COI gene spesific primers with sequences of (1) forward 5’-TTC-TCC-AAC-CAC-AAA-GAC-ATT-GGC-AC-3’ and (2) reverse 5’-ACT-ACA-TGT-GAG-ATG-ATG-CCG-AAT-3’ [9]. The COI gene was amplified in a Polymerase Chain Reaction (PCR) machine. The stages of this amplification were: an initial denaturation at 95°C for 5 minutes, followed by five denaturation cycles at 94°C for 1 minute, 45°C of annealing for 1.5 minutes and 72°C of extension for 1.5 minutes. Following this, 30 denaturation cycles at 94°C for 1 minute, 50°C annealing for 1.5 minutes, extension 72°C for 1.5 minutes, then the final extension starting at 72°C for 5 minutes [9]. The PCR product was visualized under UV light on 0.8% agarose gel immersed in EtBr solution following electrophoresis. After obtaining the targeted band, these PCR products were then sent to the First Base Laboratories, Malaysia for sequencing.
2.3. Analysis of the sequencing results

Multiple alignment analysis of COI sequences was conducted using a MEGAX software. After aligning all sequences using ClustalX method, the results were aligned with the sequence of other species belong to Accipitridae from GenBank and BOLD Systems, using Basic Local Alignment Search Tool (BLAST) analysis. Species belong to genus *Nisaetus* used as reference species were *N. nipalensis* AB843170.1, AB843171.1, AB843172.1, AB843173.1, AB843766.1, AB843767.1, AP008238.1; *N. alboniger* AP008239.1; *N. bartelsi* MTNB001-18, MTNB002-18, MTNB003-18, MTNB004-18, MTNB005-18; *N. cirrhatus* ROMC331-07; *N. philippensis* HM639912.1, those belong to genus *Spizaetus* were *S. tyrannus* JQ176245.1; *S. melanoleucus* JQ176244.1, those belong to genus *Aquila* were *A. chrysaetos* GU571738.1, GU571264.1, and outgroup belong to genus *Microhierax* were *M. caerulescens* JQ175370.1; *M. erythrogenys* JQ175371.1, JQ175372.2 from Falconidae family. The phylogenetic tree was reconstructed using Neighbour Joining (NJ) method with substitutions model using Kimura 2-parameter model [10].

3. Results and Discussion

3.1. The similarity of encoded species samples to another

The target gene of approximately 700 bp was obtained during COI gene amplification based on the electrophoresis results. The sequencing results of RR13, RR14, RR15, RR16, and RR19 attained 766, 756, 743, 746, and 745 base pairs, respectively. The results of the BLAST analysis showed that our samples were closely related to *N. philippensis*, with similarity of higher than 96 % (Table 1). The phylogenetic tree result also showed that individuals of RR13, RR14, RR15, RR16, and RR19 formed sister species clade with *N. philippensis*. The further insight into similarity between the used taxa was found in analysis of pairwise genetic distance amongst the five samples and COI sequences of another eagle species.

| No | Sample Code | Similarity (%) | Query Cover (%) | Nucleotide Differences |
|----|-------------|----------------|-----------------|-----------------------|
| 1  | RR13        | 96.19          | 89              | 26/683                |
| 2  | RR14        | 96.78          | 90              | 22/683                |
| 3  | RR15        | 96.49          | 91              | 24/683                |
| 4  | RR16        | 96.49          | 91              | 24/683                |
| 5  | RR19        | 96.49          | 91              | 24/684                |

The genetic distances amongst the five samples and another used COI sequences underlay its grouping in the intraspecific and interspecific analysis. In accordance with the intraspecific distance, the Changeable Hawk-eagle (*N. philippensis*) had very low genetic variation within the species. With a genetic distance of 0.0016 (Table 2), means that the differences amongst *N. cirrhatus* sequences observed was 0.16%. Moreover, it was obvious that interspecific genetic distance of Accipitridae family sequences had divergence of 0-0.182 which means there were differences in the amount of 0-18% (Table 3). The genetic distance between this group also yielded that *N. cirrhatus* had closely relatedness with *N. philippensis*, with divergence value of 0.038 (3.8%) and very distant with *Microhierax caerulescens* (19.6%). According to these results, COI gene sequence is appropriate to identify species because it has low variability within species (1-2%) [9] because it can be more specific to identifying species. The high variability between species especially for the both different closely related taxa also becoming the reason why COI gene sequence was used in the barcoding species [11]. Therefore, the divergence of two sequences above 2% allows the formation of species complex.
Table 2. Intraspesific genetic distance of *N. Cirrhatus* against few species from GenBank.

| Species               | Distance | Standard Error |
|-----------------------|----------|----------------|
| *Nisaetus cirrhatus*  | 0.0016   | 0.0011         |
| *Nisaetus bartelsi*   | 0        | 0              |
| *Nisaetus alboniger*  | n/c      | n/c            |
| *Nisaetus nipalensis* | 0        | 0              |
| *Spizaetus tyrannus*  | n/c      | n/c            |
| *Nisaetus philippensis* | n/c    | n/c            |
| *Spizaetus melanoleucus* | n/c | n/c            |
| *Aquila chrysaetos*   | 0        | 0              |
| *Microhierax caerulescens* | n/c | n/c            |
| *Microhierax erythrogenys* | 0  | 0              |

Table 3. Interspesisfic genetic distances of few genus in Accipitridae family.

| Taxon name               | a)  | b)  | c)  | d)  | e)  | f)  | g)  | h)  | i)  | j)  |
|--------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| a) *Nisaetus cirrhatus*  | 0.013| 0.012| 0.013| 0.015| 0.008| 0.014| 0.018| 0.017| 0.014|     |
| b) *Nisaetus bartelsi*   | 0.088| 0.005| 0.006| 0.016| 0.014| 0.014| 0.018| 0.020| 0.013|     |
| c) *Nisaetus alboniger*  | 0.086| 0.015| 0.007| 0.015| 0.013| 0.013| 0.018| 0.020| 0.014|     |
| d) *Nisaetus nipalensis* | 0.092| 0.024| 0.029| 0.016| 0.014| 0.014| 0.018| 0.020| 0.013|     |
| e) *Spizaetus tyrannus*  | 0.106| 0.115| 0.106| 0.113| 0.014| 0.012| 0.020| 0.020| 0.015|     |
| f) *Nisaetus philippensis* | 0.038| 0.093| 0.091| 0.097| 0.097| 0.013| 0.019| 0.018| 0.015|     |
| g) *Spizaetus melanoleucus* | 0.095| 0.100| 0.096| 0.100| 0.077| 0.093| 0.020| 0.019| 0.016|     |
| h) *Microhierax caerulescens* | 0.196| 0.184| 0.186| 0.182| 0.214| 0.193| 0.205| 0.013| 0.018|     |
| i) *Microhierax erythrogenys* | 0.174| 0.200| 0.202| 0.198| 0.193| 0.171| 0.182| 0.087| 0.018|     |
| j) *Aquila chrysaetos* | 0.105| 0.096| 0.102| 0.098| 0.109| 0.112| 0.110| 0.180| 0.182|     |

Taxon name: a) *Nisaetus cirrhatus*, b) *Nisaetus bartelsi*, c) *Nisaetus alboniger*, d) *Nisaetus nipalensis*, e) *Spizaetus tyrannus*, f) *Nisaetus philippensis*, g) *Spizaetus melanoleucus*, h) *Microhierax caerulescens*, i) *Microhierax erythrogenys*, j) *Aquila chrysaetos*.

Source: GenBank.

3.2. Phylogenetic tree of Changeable Hawk-eagle

The topology of phylogenetic tree using Neighbour Joining (NJ) method constructed from five samples, RR13, RR14, RR15, RR16, and RR19 within the same species, formed complex clades with different bootstrap value. This phylogenetic tree also confirmed that *N. cirrhatus* was closely related to *N. philippensis* as sister species, with a high bootstrap value of 99. *N. cirrhatus* and *N. philippensis* forms a monophyletic group along with *S. tyrannus* and *S. melanoleucus*, yet has a low bootstrap value (40) (Figure 1). Based on this result, complex clades of *N. cirrhatus* was suspected to be a cryptic species due to the formation of haplogroup. Its low genetic distance within *N. cirrhatus* species (0.16%) indicated that an alteration still occurs in genetic within species due to the potential environmental changes.

The Changeable Hawk-eagle is found at latitudes of 1500-2200 meters above sea level (masl) in open areas such as savanna and forest which is close to water sources [12]. The wide home range (33-155 km²) of Changeable Hawk-eagle may affect its competency to breed, the availability of food resources, and its shelter presence [13]. Therefore, habitat characteristics may also influence its reproductive success.
The development of reproductive isolation between the populations of Changeable Hawk-eagle is the key event to speciation process [14]. Despite of unavailable information of our samples origin, these Changeable Hawk-eagles has small genetic divergence because it was obtained from one place. The gene tracking using COI sequences of these Changeable Hawk-eagles could be a rapid solution to identify this species, because COI gene was relatively stable against the environmental changes [9] so it would be more specific than others. The erratic haplotype distribution in N. cirrhatus could also be the consequence of incomplete lineage sorting [8]. Therefore, even the smallest genetic divergence within this species can be considered to define the genetic peculiarities which maintained as an answer to specific environmental changes [8].

The segregated habitat of Changeable Hawk-eagle on Java and other Islands could isolate one subspecies from another and form a local adaptation that could be more distinct over time. Due to its wide geographical range and the difference in prey selection within the two sexes of Changeable Hawk-eagle [15], this species has a major role in maintaining the ecosystems. In regards of the less information and genetic data of N. cirrhatus, this research can be considered as a preliminary study before the species becomes rare. Through the study of Phylogenetic Species Concept, genetic diversity can be used to reveal the phylogenetic relationship between species in order to determine management unit (MU) and evolutionary significant units (EU) which is in turn become essential for conservation [16].

4. Conclusion
The COI gene sequence of our samples (RR13, RR14, RR15, RR16, and RR19) were confirmed as Nisaetus cirrhatus. The NJ tree showed that this species formed a monophyletic clade with smaller branches indicating a species complex. The genetic distance indicated that Nisaetus cirrhatus formed a low genetic variation and was related to Nisaetus philippensis.

Figure 1. Phylogenetic tree of Nisaetus cirrhatus and other species in Accipitridae family.
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