A phylogenetic analysis of Crested Serpent Eagle (*Spilornis cheela*) based on *cytochrome-c oxidase subunit I* (*COI*): a stepping stone towards genetic conservation of raptors in Indonesia

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Abstract. As one of the most widespread raptors in Asia, Crested Serpent Eagle (*Spilornis cheela*) are genetically varied. However, due to the limited studies on genetic aspects, the genetic difference based its geographic distribution are difficult to define. A thorough phylogenetic analysis is needed to create a coherent taxonomic definition for the sake of conservation; starts with phylogenetic analysis based on *cytochrome-c oxidase subunit I* (*COI*) gene as the standardized genetic marker to define species. This research was aimed to resolve within species phylogenetic diversity and to explain the relationship between species based on COI gene. Total DNA was isolated from blood samples. The BLAST confirmed PCR amplified *COI* gene fragments were visualized on 0.8 agarose gel. Sequencing was carried out by FirstBase Laboratory Malaysia. Phylogenetic analysis was done using AMOVA and MEGA7 software in Neighbor Joining method with Kimura-2 parameter. Reconstructed phylogenetic tree confirmed the divergence between three samples and another *S. cheela* from other regions respective to their geographic distributions. There was 0,014 genetic distance between samples and *S. cheela* and 0,015 between *S. cheela* and other species among genus *Spilornis*. This research also revealed that *S. cheela* and *S. holospilus* were not significantly varied.

1. Introduction

Becoming one of the most widespread birds of prey in Indomalayan region [1], Crested Serpent Eagle (*Spilornis cheela*) has raised several questions regarding their evolution. This species is renown to be habitat generalist, which means they can adapt to a wide array of ecosystem from tropical rainforest,
savanna to mangrove [2], and are abundant in almost all kinds of forest ecosystem in Indomalayan region [1]. Noting from their adaptability and abundance, hence this species is categorized as Least Concern by IUCN RedList [3] despite the fact their population is in decline. In Indonesia, this decline is mostly caused by anthropogenic activities such as agricultural expansion in their natural habitat which fragments their habitat and poaching to fulfil the demand for wildlife trafficking [4]. This intrusion combined with the geographical condition of Southeast Asia, particularly Indonesia, will eventually draw barriers between subpopulations which can limit the connectivity, hence also the gene flow from one subpopulation to another [5]. With this threat looming over this species, the habitat generalist has the potentials to be habitat specialists in the longer term due to local adaptations in response to the changing landscape [6]; and with the fragmented habitat, the inbreeding depression will be the main threat to the genetic variation; spiraling this species to local extinction vortex [7].

Despite the race against local extinction in Indonesia, the genetic aspect of this species is still poorly studied. Studies conducted on this species includes their spacing pattern [8], habitat preference [9], their subspecies reports in many regions in Southeast Asia as well as Indonesia [10], and their breeding behaviour [11]. Recent studies also indicate that due to their wide distribution and within species diversity, Crested Serpent Eagle often faces taxonomic confusion whether or not some subpopulations of this species should be categorized as a separate species [12]. The uncertain status of this species has encouraged researchers to delineate them based on molecular markers [13]. The genetic records on this species can be found on GenBank database, but these genetic data came from Crested Serpent Eagle which are distributed in China [1], in Japan [14] and Nepal [13]; but reports on genetic aspect of Indonesian Crested Serpent Eagle in Indonesia are still scarce. Moreover, genetic studies on this species mostly focus on their taxonomic status in order to define their lineage on the Tree of Life [14]. A clear taxonomic status is indeed essential to determine the evolutionarily significant units (ESU) as well as Management Units (MU) in conservation endeavor [15]. However, understanding the genetic diversity and how a species evolve is as essential as determining taxonomic status [16] as understanding the underlying process which constitute their diversity will also be useful for long term conservation programs [17].

Taking the genetic process into account, as well as the potentials of local adaptation in Indonesian archipelago, a thorough phylogenetic analysis and the phylogeographical study of Crested Serpent Eagle in Indonesia is needed. The Crested Serpent Eagle in Indonesia may form several subspecies which each of them is endemic in their locally distribution. This study focuses on the phylogeny of Crested Serpent Eagle to describe the lineage of Crested Serpent Eagle in Indonesia using a DNA barcode Cytochrome c Oxidase Subunit I (COI). COI is a relatively short sequence (~ 640 – 780 bp) of mitochondrial genome that is used as a standardized molecular marker to identify species up to the species level [18]. The use of DNA barcode sequence in phylogeny has been known to be robust in delineating various species and describe their phylogenetic diversity [18].

The Crested Serpent Eagles used as samples in this study were retrieved from Cikananga Wildlife Centre with their subspecies are unknown yet. This uncertainty about their subspecies can lead into suspicion about the marital behavior for each subspecies, since they are endemic in their local distribution. This can be used as consideration for the releasing programs, whether it will be in situ or ex situ. Therefore, this study was aimed to describe the phylogenetic diversity of Crested Serpent Eagle in Indonesia as a stepping-stone for further studies on genetic level to answer the question on how this species adapt to various kind of ecosystem because of the habitat fragmentation then formed various subspecies; and eventually answer the question on how we can maintain their genetic diversity through conservation genetics.

2. Methods

2.1. Collection of Samples and Data Acquisition

This study was conducted from March to May 2019 in the Genetic Regulation Laboratory and Biotechnology Division of Central Laboratory of Mineral and Advanced Material, State University of Malang. Three individuals Crested Serpent Eagle used in this study were from Cikananga Wildlife Centre.
Centre in Sukabumi District, encoded as RR06, RR07, and RR08 (assumed to be captured from Borneo and Java). Another S. cheela sequences were mined from GeneBank of National Center for Biotechnology Information (NCBI) with accession number respectively JQ176242.2, JQ176242.1, AB843765.1, AB843764.1, AB843763.1, AB843762.1.

Distribution of samples and S. cheela COI gene sequences mined from gene bank showed below in Figure 1. 2 sequences obtained from research report conducted in Central Luzon, Philippines with accession number JQ176242.2 and JQ176242.1 in gene bank. Another 4 sequences are based from report conducted in Okinawa, Japan with GeneBank accession number AB843765.1, AB843764.1, AB843763.1, and AB843762.1.

![Figure 1](image-url)

**Figure 1.** The red dot showed the distribution of *Spilornis cheela* COI gene sequence used in this report. 3 samples are from Cikananga, West Java, Indonesia; 2 sequences from Central Luzon, Philippines; and 4 sequences from Okinawa, Japan.

### 2.2. Isolation of DNA and Amplification of COI gene

We used blood samples taken from pectoral subclavian veins of each individual of Crested Serpent Eagle by a veterinarian in Cikananga Wildlife Center. The DNA materials were then stored in 1000 μl of 98% ethanol. The DNA extraction was performed using DNeasy Blood and Animal Tissue Kit (Qiagen, Cat. No. 69504). Amplification of COI gene through PCR was performed using COI primers: (1) forward 5’- TTC TCC AAC CAC AAA GAC ATT GGC AC-3’ and (2) reverse 5’ ACT ACA TGT GAG ATG ATT CCG AAT-3’ [19]. We used PCR cycle parameters as follows: initial denaturation of 95°C for 5 minutes, followed by five cycles of 94°C denaturation for 1.5 minutes, 54°C annealing for 1.5 minutes and 72°C extension for 1.5 minutes. Following this, 30 cycles of 94°C denaturation for 1.5 minutes, 57°C annealing for 1.5 minutes, 72°C extension for 1.5 minutes, then the final extension at 72°C for 5 minutes [19]. Amplification results were then visualized by performing electrophoresis in 0.8% agarose gel. After the targeted band (~700 bp length) was visible, the samples were sent to First Base Laboratories Malaysia for sequencing process.

### 2.3. Analysis of the Sequencing Results

Sequencing results were read using FinchTV software (©Digital World Biology LLC), trimmed using MEGA7, and combined using DNA Baser to create a consensus sequence (Heracle BioSoft SRL). Multiple alignments were performed using ClustalX2 among forward and reverse sequence for each sample to produce consensus sequence. Similarity index of consensus sequence from samples obtained from BOLDsystem database (using BOLD system alignment browser) and from GeneBank (using Basic Local Alignment Search Tool or BLAST analysis). Pairwise genetic distance was conducted among
three encoded samples and *S. holospilus* as the closest relative. The intraspecies and interspecies genetic distance analysis in comparison with other members of the *Spilornis* genus using MEGA7. We ran AMOVA in order to examine the genetic variance within species and among species. The phylogenetic tree analysis was conducted using MEGA7 with the neighbor-joining (NJ) method according to Kimura 2-parameter [20] with three encoded samples and another *Spilornis cheela* sequences as shown above. Another species’ sequence provided from GeneBank of National Center for Biotechnology Information (NCBI) such as *S. holospilus* (HM639910.1, HM639911.1), *Terathopius* from subfamily Circaetinae (*Terathopius ecaudatus* KX012845.1, KX012843.1, KX012794.1), *Haliastur* genus as representative from family Accipitridae (*Haliastur indus* HM639876.1 and HM639875.1), also *Falco* genus from Falconiformes family as outgrup (*Falco peregrinus* GU571881.1 and GU571880.1) were used as reference species.

3. Results and discussion

3.1. The similarity of encoded species samples and reference species

Morphological study of samples RR06, RR07, and RR08 shown average number of its weight was 1.13 kg, total length was 34 cm, and 1 m of wingspan. The COI gene amplified from samples RR06 and RR07 showed 764 bp length, and sample RR08 showed 759 bp length (Figure 2). Identification and similarity result from BOLDSystem showed that each sample were indeed indicated as *Spilornis* genus with 97.5% similarity. Each sample was indicated as *S. cheela* with similarity above 97% (Table 1). Similar results according to BLAST analysis from GeneBank suggest that samples were highly similar with *S. cheela* as shown in Table 2.

![Figure 2](image)

Table 1. The similarity of samples against *S. cheela* based from BOLDSystem

| No | Sample Code | Similarity (%) |
|----|-------------|----------------|
| 1  | RR06        | 97.49          |
| 2  | RR07        | 97.49          |
| 3  | RR08        | 97.64          |
3.2. The Genetic Distance of Crested Serpent Eagle and Other Species

The pairwise genetic distance analysis of the samples showed that *S. holospilus* HM639910.1, HM639911.1 were the closest relative with *Falco peregrinus* GU571881.1 and GU571880.1 as an outgroup. Samples RR06, RR07, and RR08 showed 0.000 genetic distance, meaning that the samples had no variance in their COI gene sequences. That number also indicated that the samples belong to the same species. Furthermore, the pairwise genetic distance of each samples compared to another *S. cheela* indicated genetic distance of 0.024 and 0.029 compared to *S. holospilus*. This means the similarity between the said taxa was 97.6% and 97.1% (Table 3). This percentage indicated that the samples belonged to one metapopulation of species, as they showed low rate of divergence, ranging from 0.6-2.0%, while divergence values between species were ordinarily greater than 3% (0.03) [21]. This result indicated that samples were belonged to *Spilornis cheela* metapopulation as a species.

This confirmed state of the three samples as Serpent Crested Eagle (*S. cheela*) according to their pairwise genetic distance was then used as a basis for generating intraspecies and interspecies genetic distance. Based on intraspecies genetic distance of *S. cheela* samples against another *S. cheela* shown genetic distance of 0.014 and genetic distance between *S. cheela* samples compared to all of *Spilornis* species was 0.015, which considered as low distance but enough to formed gap between subspecies. The results indicated that Crested Serpent Eagle in Asia developed genetic variations among subpopulations of *S. cheela* due to the geographical barriers that limit gene flow between subpopulations.

| Table 2. The similarity of samples against *S. cheela* sequences from GeneBank databases by BLAST Analysis |
|---------------------------------|---------------------------------|------------------|------------------|
| No | Sample Code | Similarity (%) | Query Covers (%) |
|----|-------------|----------------|------------------|
| 1  | RR06        | 96             | 97.25            |
| 2  | RR07        | 96             | 97.25            |
| 3  | RR08        | 97             | 97.40            |

| Table 3. The pairwise distance among samples and another member of *Spilornis* genus |
|---------------------------------|---------------------------------|------------------|
| Species name | a) | b) | c) | d) | e) | f) | g) | h) | i) | j) | h) |
|---------------------------------|------------------|------------------|
| a) RR06 | 0.000 | 0.000 | 0.000 | 0.007 | 0.007 | 0.006 | 0.006 | 0.006 | 0.007 | 0.007 | 0.015 |
| b) RR07 | 0.000 | 0.007 | 0.007 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.007 | 0.007 | 0.015 |
| c) RR08 | 0.000 | 0.000 | 0.007 | 0.007 | 0.006 | 0.004 | 0.004 | 0.004 | 0.002 | 0.002 | 0.015 |
| d) *S. cheela* JQ176242.2 | 0.024 | 0.024 | 0.024 | 0.000 | 0.004 | 0.004 | 0.004 | 0.004 | 0.002 | 0.002 | 0.014 |
| e) *S. cheela* JQ176242.1 | 0.024 | 0.024 | 0.024 | 0.010 | 0.004 | 0.000 | 0.000 | 0.000 | 0.002 | 0.002 | 0.014 |
| f) *S. cheela* AB843765.1 | 0.024 | 0.024 | 0.024 | 0.010 | 0.010 | 0.000 | 0.000 | 0.000 | 0.004 | 0.004 | 0.015 |
| g) *S. cheela* AB843764.1 | 0.024 | 0.024 | 0.024 | 0.010 | 0.010 | 0.000 | 0.000 | 0.000 | 0.004 | 0.004 | 0.015 |
| h) *S. cheela* AB843763.1 | 0.024 | 0.024 | 0.024 | 0.010 | 0.010 | 0.010 | 0.000 | 0.004 | 0.004 | 0.004 | 0.015 |
| i) *S. cheela* AB843762.1 | 0.024 | 0.024 | 0.024 | 0.010 | 0.010 | 0.010 | 0.010 | 0.000 | 0.004 | 0.004 | 0.015 |
| j) *S. holospilus* HM639910.1 | 0.029 | 0.029 | 0.029 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | 0.014 |
| k) *S. holospilus* HM639911.1 | 0.029 | 0.029 | 0.029 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | 0.002 | 0.012 |

However, The AMOVA analysis suggested that there was no significant difference between groups of subpopulations. The percentage of variance between groups of localities showed very low variance of 54% variance with p-value of 0.3 (Table 4). When the percentage is below 0, this means that each group has low FST value. The low FST value means that each group has low genetic distance, in other words, each group from each locality are not significantly different from each other genetically to be
categorized as distinct species. This low FST was shown in Figure 3. Nevertheless, to confirm this theory, more COI gene of *Spilornis cheela* from across Asia and especially Indonesia is needed.

**Table 4.** The AMOVA result between groups in subpopulation

|                  | Sigma     | %          |
|------------------|-----------|------------|
| Variations between group | -0.4517857 | -54.96017  |
| Variation between samples within group | 0.7261905 | 88.34178   |
| Variation within samples | 0.5476190 | 66.61839   |
| Total            | 0.8220238 | 100.00000  |

**Figure 3.** The plot describing the genetic variations between locality groups suggests that there is no significant difference between groups of localities.

Analysis of interspecific genetic distance was conducted to determine relationship amongst *S. cheela* with other closely related taxa (Table 5). Result shown that *Spilornis* genus had genetic distance number 0.128 compared to another member of family Accipitridae (genus *Therathopius* and *Haliastur*). Result also shown genetic distance of 0.213 compared to *Falco peregrinus* as an outgrup. Those number were high enough to formed another cluster between taxa [22].

**Table 5.** The AMOVA result between groups in subpopulation

|                  | Spilornis | Circaetinae | Outgrup |
|------------------|-----------|------------|---------|
| Spilornis        | 0.128     |            |         |
| Circaetinae      |           | 0.213      | 0.193   |
| Outgrup          |           |            |         |

3.3. Phylogenetic tree of Crested Serpent Eagle

The phylogenetic tree reconstruction showed that the three samples form a monophyletic group with other Crested Serpent Eagle species with the bootstrap value of 100. In this monophyletic clade, the samples from Indonesia formed a different branch from the rest of the group with bootstrap value of 83. The tree also shown that *S. holospilus* (HM639910.1, HM639911.1) belonged in one same clade with *S. cheela* (JQ176242.2, JQ176242.1) and both of them are branched from another *S. cheela*
(AB843765.1, AB843764.1, AB843763.1, AB843762.1). The topology of the phylogenetic tree supported the notion that there was genetic variation within Crested Serpent Eagle but this genetic variation was not significant enough to diverge this species into separate groups as the pairwise genetic distance and AMOVA results suggested.

Another COI gene of S. cheela used in the tree were coming from Central Luzon, Philippine with accession code JQ176242.2, JQ176242.1 [14] and from Okinawa, Japan (AB843765.1, AB843764.1, AB843763.1, AB843762.1) [23]. Meanwhile the samples used here were from Indonesia (from Cikananga, West Java). This tree result was intriguing because S. holospilus accession code HM639910.1, HM639911.1 which were from Davao, Philippines [24] positioned in the middle of another S. cheela, indicated that they were not a different species but a subspecies from S. cheela. All of that Spilornis genus were in one group with Therophilus as subfamily Circetaeinae, with Haliastur as one family Accipitridae and yet formed another branch from outgroup Falco peregrinus (Figure 4).

Crested Serpent Eagle usually grouped in the genus Spilornis, which have a large distribution region in Asia, India, Andaman Islands, and Indo-Malayan areas. Most of them distributed in Himalayan, Pakistan, Kashmir, Nepal, China, Palawan, and Indonesia [24; 8; 1]. Crested Serpent Eagle formed 26 subspecies based on their biogeographic areas and Indonesia alone, there were 16 known subspecies of S. cheela which all of them recognized as endemic species such as Bawean Serpent Eagle (S. c. bawecaus), Simeulue Serpent Eagle (S. c. abotti), Nias Serpent Eagle (S. c. casturinus), Mentawai Serpent Eagle (S. c. sipora), Natuna Serpent Eagle (S. c. natunensis), Sulawesi Serpent Eagle (S. c. rufipectus) [26]. According to this study, S. cheela samples were located in the same branch with another S. cheela and S. holospilus, but formed different clade. This was caused by their different distribution area formed some genetic variation between them, so we concluded that samples were another subspecies from S.
Subspecies could still interbreed at their boundaries so that genes and morphological characters could flow between them [27]. So, we assumed that samples were subspecies from *Spilornis cheela* endemic either from Borneo or Java due to their captured place. Although *S cheela* are widely distributed among Asia continent, they formed some genetic variation for each sequence taken. Due to their taken place which are all an archipelago (Indonesia, Philippines, and Japan), it is possible that there are some landscape barrier for them so cannot interact with another group. Broadly distributed species are more likely to experience population subdivisions and greater probabilities of developing molecular subclades because their densities are often relatively uniform across habitats and thus exposed to local threats [28], so they became locally adapted for each island.

However, based on their high variation, several taxa been described as separate species [10]. Like *S. holospilus* which grouped as a different species [29] but based on the tree formed they were still formed one clade with *S. cheela*. This is why further research based on the COI gene of *S. cheela* and more samples are needed from across Asia (mainland and archipelago) and Indonesia to determine genetic diversity among them. In this study, *S. cheela* and *S. holospilus* considered as one group by their position in phylogeny tree, their genetic distance was not significantly different, and their similarities are considered high. Morphologically, they are not far different, they have brownish color and a black crown with numerous white spots in their bodies, and black bands on their wings and tail feather, short toes, meanwhile *S. holospilus* are smaller than the average *S. cheela* and they have neater and more define white spots over their bodies and wings.

The intraspecific and interspecific distance among genus *Spilornis* showed number 0.014 and 0.015 which considered as low in their genetic variation. This is happened due to their restricted distribution which were found only in the Old World [13], considered the sequences and samples are taken from Asian archipelago. Meanwhile the distance among them and the Circaetinae subfamily showed number 0.128, considered as high due to their polyphyletic subfamily from Eutrochis and Dryotriorchis. This deepest split within the snake eagles extremely linked to their geographic distribution where Indomalayan species might form separate clades for their subspecies.

*Spilornis* subspecies are endemic due to their biogeographic distributions and there is no complete record about their genetic records yet, include that 26 subspecies in Indonesia. Their populations are declining because they faced some threats such habitat fragmentation, hunting and illegal trading, illegal logging, agricultural expansion, and the use of pesticide in their food chain [4; 30]. This change of habitat can be responded by local adaptation of each group [31]. This situation highlights the importance of assessing the phylogenetic history and genetic distinctiveness of the *Spilornis* species. We suggest relationships of all the snake eagle subspecies should be further explored, based on their biogeographic distribution, with increased sampling from around Asia to inform attempts to conserve these unique and relatively little-known taxa. Molecular phylogenetics used to test biogeographic hypotheses of speciation among various organisms. It enables one to assess genetic similarity directly across vast distances, or those as narrow as the width of a river, and to test how these potential barriers affect gene flow and ultimately phylogenetic relationships and taxonomy [32].

Facing some conservation goals, a reliable diagnosis of the taxonomic status of populations is essential. An unrecognized species may become extinct due to a lack of information, including genetic information, which can lead to a lack of protection. A taxonomic unit as detailed as the species level is usually prioritized in conservation measures as an evolutionarily significant unit [5]. As a result, the phylogenetic and genetic diversity affects conservation more than any other disciplines. This knowledge of genetic diversity of *S. cheela*, especially in Asia can be used as a base for management decision to maintain their evolution process. However, as long as maintenance of evolutionary processes in populations is a goal of management, rather than the maintenance of variants alone [15].

4. Conclusion
The COI gene sequences obtained from three samples encoded as RR06, RR07 had 764 bp length, and RR08 had 759bp length. According to the phylogenetic analysis, Crested Serpent Eagle RR06, RR07, and RR08, was confirmed as one species *Spilornis cheela* with subspecies still unknown but assumed
endemic from either Borneo or Java and varied with another S. cheela due to their geographic distribution. More samples of S. cheela COI gene from Asia (mainland and archipelago) are needed to obtain, improve and complement phylogeny tree, also to determine Crested Serpent Eagle’s genetic diversity, also to determine Crested Serpent Eagle taxa position, particularly in Indonesia. This DNA Barcoding obtained in this study can be used to investigate the genetic structure of populations of Spilornis and subsequently improving their breeding and maintaining their evolutionary process in population.

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