Genetic identification of Javan hawk-eagle (*Nisaetus bartelsi*) from Indonesia using mitochondrial COI gene

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Abstract. *Nisaetus bartelsi* is a native hawk from Java which its genetic information is not commonly understood yet. As a part of the conservation effort to maintain genetic diversity of this endangered species, this research aimed to obtain COI gene sequences from five individuals of *N. bartelsi* to confirm its position in the phylogenetic tree. DNA isolation from 5 *N. bartelsi* blood sample was performed and its COI gene sequence was amplified, sequenced, and used to reconstruct phylogenetic tree using MEGA6 with several other members of *Nisaetus*, *Aquila*, and *Sagittaridae* family. Furthermore, the intraspecific distance between 5 *N. bartelsi* samples and interspecific distance with other species were calculated using MEGA6. The result suggested that all five individuals belonged to the species Javan hawk-eagle (*N. bartelsi*) and were closely related to the Blyth's hawk-eagle (*Nisaetus alboniger*). The DNA barcoding of the Javan hawk-eagle conducted in this study is a stepping stone to conservation efforts for the Javan hawk-eagle.

Keywords: conservation, cytochrome-c oxidase subunit I, DNA barcoding, Javan hawk-eagle

INTRODUCTION

The Indonesian archipelago is renowned for its enormous species endemism due to its geographical condition. One of the endemic species of Indonesia is the Javan hawk-eagle (*Nisaetus bartelsi*) (Nijman & Sözer, 1998), a diurnal bird of prey living solely in the tropical rainforest of Java (Van Balen *et al.*, 2000). This species mostly inhabits primary rainforest (Van Balen *et al.*, 2001) and is also frequently seen in secondary or even production forests in Java (Syartinilia & Tsuyuki, 2008) ranging from sea level to high mountains, with the most frequent appearance found at 500-1000 m. Furthermore, the estimated average home range of one pair is approximately 400 ha (Gjershaug *et al.*, 2004). The preferred diet of Javan hawk-eagles varies from small mammals to birds and reptiles (Prawiradilaga, 2006).

Javan hawk-eagles' resemblance to the

mythical creature Garuda, which is also the national emblem of Indonesia, has made this species a prominent focus for avian conservation in Java (Van Balen *et al.*, 2000). Furthermore, since the declaration of this species as a National Rare/Precious Animal of Indonesia in 1992 (Widyastuti, 1993), public awareness about the need to preserve their habitat, along with natural resources that support it, has increased. However, this rising awareness has also increased its demand in illegal wildlife trade since in Javanese culture, bird keeping, especially the keeping of expensive and rare birds, is a way to exhibit one's social status (Nijman, 2009).

The population of this species is decreasing despite the legal status of the Javan hawk-eagle and the ongoing conservation efforts (IUCN, 2016; BirdLife International, 2016). The estimated

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population of Javan hawk-eagles throughout the island of Java is around 600-900 individuals, which roughly equals 300-500 mature individuals (IUCN, 2016). The reason for this decrease, besides illegal wildlife trade, is deforestation (Sozer *et al.*, 1998; Collar *et al.*, 2001; Nijman, 2009), which eventually leads to habitat fragmentation (Van Balen *et al.*, 2000). As a result, Javan hawk-eagle populations throughout Java are severely fragmented and, most likely, isolated from each other (Van Balen *et al.*, 2000). Meanwhile, isolated populations are vulnerable to inbreeding depression, which affects birth weight, survival, reproduction and disease resistance due to the loss of genetic diversity within the population; populations with these conditions are prone to extinction (Keller & Waller, 2002).

In response to the race against extinction caused both by human disturbance and inbreeding depression, conservation efforts at the molecular level are necessary in order to save any records of the species, including their genetic data, and to maintain the phylogenetic diversity of the Javan hawk-eagle (Frankham, 2003). Taxonomic or phylogenetic distinctiveness is one determining factor in establishing conservation priorities for threatened species, like the Javan hawk-eagle (IUCN, 1980).

In the previous phylogenetic study based on mtDNA sequences *Cyt-b* and non-coding control region, *Nisaetus bartelsi* is sister species to *Nisaetus alboniger* forming a distinct clade apart from *Nisaetus kelaarti* and *Nisaetus nipalensis*; both *Nisaetus bartelsi* and *Nisaetus alboniger* belong to the Asian hawk-eagle monophyletic cluster apart from their relatives *Spizaetus* genus in South America (Haring *et al.*, 2007). Due to the short *cyt-b* sequence, the phylogenetic tree in the previous study resulted in low bootstrap values in the phylogenetic tree especially in the deeper nodes, indicating that further genetic analysis using higher resolution genetic markers is necessary (Haring *et al.*, 2007). Further study based on mitochondrial genes *Cyt-b* and 5 nuclear loci also support that *Nisaetus bartelsi* is sister species to *Nisaetus alboniger* and both taxa form a separate clade from *Nisaetus nipalensis* (Lerner *et al.*, 2017). Nevertheless, to clarify the phylogenetic distinctiveness of Javan hawk-eagles, a database of the COI gene is needed.

The cytochrome-c oxidase subunit I (COI)

gene is a short sequence of mitochondrial DNA (648-780 bp) that serves as a standardized molecular marker, or commonly known as a DNA barcode, for biological identification with precision up to the species level due to its low variability (1-2%) and low rate of amino acid changes (Hebert *et al.*, 2003; Hebert *et al.*, 2004; Hajibabaei *et al.*, 2007). DNA barcodes can provide a clarification of the genetic distinctiveness of a species by confirming the species name of the taxa based on its unique COI gene sequence (Hajibabaei *et al.*, 2007). To date, however, there is still a lack of reports in GenBank on the genetic data, especially the COI gene, of Javan hawk-eagles living in the primary forests on the island of Java.

A thorough phylogenetic analysis is needed to trace the genetic diversity of this species and to address the lack of genetic data for the Javan hawk-eagle. This is needed to inform conservation programs that aim to preserve the genetic diversity of distinguishable taxonomic units (Lerner & Mindle, 2005). This should be done since concerns about the evolutionary information loss of a species are one of the basic motivations of nature conservation itself (Winter *et al.*, 2012). The COI gene sequence of the Javan hawk-eagle can provide deep insight into the phylogenetic diversity of this species, since it is used as a base to reconstruct a phylogenetic tree and is highly useful in estimating the relationship amongst species, as well as its evolution (Hajibabaei *et al.*, 2007; Winter *et al.*, 2012). Furthermore, the phylogenetic diversity that is defined with DNA barcode sequence data may serve as the most important measure for comparing genetic diversity and establishing a protected habitat across the landscape of Javan rainforests. Additionally, as the DNA barcode database library contains genetic data of species from across the globe, a comparative measure of phylogenetic diversity can serve as a standard metric for conservation assessment (Kress *et al.*, 2015).

The Javan hawk-eagle belongs to the family Accipitridae, along with the other members of genus *Nisaetus*, such as *N. nipalensis*, *N. alboniger*, *N. cirrhatus*, *N. kelaarti*, and *N. floris*. By comparing the COI gene sequence of *N. bartelsi* with COI genes from the rest of the genera *Nisaetus* and *Spizaetus*, we can determine the position of *N.*

bartelsi in the phylogenetic tree. Accordingly, this research aims to obtain the COI gene sequences from 5 individuals of Javan hawk eagle (*Nisaetus bartelsi*) to confirm this species position in the phylogenetic tree.

MATERIALS AND METHODS

Collection of samples. This research was conducted from December 2016 to February 2017 in the Genetic Regulation Laboratory and Biotechnology Division of Central Laboratory of Mineral and Advanced Material, State University of Malang. Javan hawk eagle's individual which used in this study were from Eco Green Park in Batu City (two individuals; encoded as FM01 and FW01), Cikananga Wildlife Centre in Sukabumi District (two individuals, encoded as RR21 and RR24), and Animal Sanctuary Trust Indonesia (ASTI) in Bogor City (an individual; encoded as RR04). The morphological data was based on morphometry and some supporting profiles provided by Cikananga Wildlife Centre, ASTI and Eco Green Park.

Isolation of DNA and amplification of COI gene. Materials of DNA which used in this study were blood samples taken from pectoralis subclavian veins of each Javan hawk-eagle individual, which were stored in 1000 µl of absolute alcohol. Each blood sample was collected by veterinarians in Eco Green Park, Cikananga Wildlife Center, and ASTI respectively. Samples were sent to the laboratory mentioned above for DNA isolation and COI gene amplification.

Isolation of DNA was performed using DNeasy Blood and Animal Tissue Kit (Qiagen, Cat. No. 69504). Amplification of COI gene 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' (Zein & Prawiradilaga, 2013). COI gene was amplified through polymerase chain reaction (PCR) which consisted of an 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 (Zein & Prawiradilaga, 2013). Amplification results were then visualized by performing electrophoresis in 1% agarose gel. After the targeted band of approximately 700 bp was visible, the samples were then sent to First Base Laboratories Malaysia for sequencing.

Analysis of the sequencing result. Sequencing results (in the form of chromatograms of forward and reverse sequence for each sample) were read using FinchTV software (©Digital World Biology LLC), trimmed using MEGA6 based on the read peaks, 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 and among encoded samples to produce consensus sequence. Multiple alignment analysis was also used to find the similarity index of consensus sequence from samples against *N. alboniger* as the same genus from BOLDsystem database (using BOLD system alignment browser) and *S. alboginer* (accession code: AP008239.1) and *S. nipalensis* (accession code: AP008238.1) as one family from GeneBank (using Basic Local Alignment Search Tool or BLAST analysis).

Pairwise genetic distance and similarity index were conducted among five encoded samples, *S. alboniger* as the closest relative and *Sagittarius serpentarius* as outgroup species. Intraspecific and interspecific genetic distance in comparison with the members of the *Nisaetus* genus was conducted using MEGA 6 (Tamura *et al.*, 2013).

The phylogenetic tree analysis was conducted using MEGA 6 with the neigh-borjoining (NJ) (Nei & Saitou, 1987) method according to Kimura 2-parameter distance with five encoded samples and some species provided in GeneBank of National Center for Biotechnology Information (NCBI) for comparison. The species that included for the reference species were in the *Nisaetus* genus (*N. Alboniger* accession AP008239.1, *N. cirrbatus* ROMC331-07, *N. tyrannus* JQ176245.1, *N. melanoleucus* JQ176244.1, *N. philppensis* HM639912.1, *N. nipalensis* AP008238.1, AB843766.1, AB843173.1, AB843767.1, AB843172.1, AB843171.1, AB843170.1), *Aquila*

genus (*Aquila chrysaetos* GU571264.1 and GU571738.1), and outgroup from Sagittariidae family (*Sagittarius serpentarius* U83776.1).

RESULTS AND DISCUSSION

The similarity of encoded samples' species and another. The morphological study conducted on the sample FW01 and FM01 showed that the individuals were 2-3 years old Javan hawk-eagles (*Nisaetus bartelsi*) or were categorized as juveniles along with the sample RR04, RR21, and RR24. The COI gene amplification and electrophoresis results suggested that the targeted gene of approximately 700 bp was obtained. The sequencing results of the five samples, with the code names FM01, FW01, RR04, RR21, and RR24, showed 744, 742, 734, 724, and 741 base pairs, respectively (Figure 1). The identification results from BOLDSystem indicated that each sample was closely related to *N. alboniger*, with a similarity above 98 % (Table 1) as there was no similarity at the species level to any sample available in the system due to the lack of genetic information on this species, especially for COI gene. The tree-based identification performed in BOLDSystem also revealed the same results. FM01, FW01, RR04, RR21, and RR24 each formed a sister species clade with *N. alboniger*. However, further analysis using pairwise genetic distance amongst the five samples and *N. alboniger* were necessary to gain further insight into the similarity between the two closely related taxa. According to Table 2, BLAST results of the Javan hawk-eagle COI gene sequence with the GenBank database suggests that the COI gene sequence

with the highest similarity was *Spizaetus alboniger*, with a query coverage of 99% and a similarity of 98%.

Figure 1. The consensus of forward and reverse sequence of the COI gene of samples FM01, FW01, RR21, RR24, and RR04, with lengths of 744 bp, 742 bp, 734 bp, 742 bp, and 741 bp respectively.

Table 1. The similarity of javan hawk-eagle from samples against *N. alboniger*.

No	Sample code	Similarity (%)
1	FW01	98.35
2	FM01	98.35
3	RR04	98.30
4	RR21	98.35
5	RR24	98.51

Table 2. The similarity of *N. bartelsi* from samples against *Spizaetus* genus.

No	Species	Accession code	Query cover (%)	Similarity (%)
1	<i>Spizaetus alboniger</i>	AP008239.1	99	98
2	<i>Spizaetus nipalensis</i>	AP008236.1	98	97

The genetic distance of Javan hawk-eagle and other species. According to the pairwise genetic distance of the five samples, with *S. alboniger* AP008239.1 as the closest relative and *Sagittarius serpentarius* U83776.1 as an outgroup, samples FM01, FW01, RR04, RR21, and RR24 had a

pairwise genetic distance of 0.000, meaning that all samples had no difference in their COI gene sequences. The genetic distance of 0.000 for each sample thus indicated that all samples belonged to the same species. Furthermore, the pairwise genetic distance of each of the five individuals of

N. bartelsi compared to *N. alboniger* or *S. alboniger* AP008239.1 supported the previous result by showing a genetic distance of 0.021; in other words, the similarity of the said taxa was 97.9% (Table 3), meaning that the five samples and *S. alboniger* AP008239.1 were sister species since genetically distinct but closely related species show low divergences, ranging from 0.6-2.0%, while divergence values between species are ordinarily greater than 3% (0.03) (Hebert *et al.*, 2003).

The confirmed status of the five samples as Javan hawk-eagles (*N. bartelsi*), according to their pairwise genetic distances, became the ground for the grouping in the intraspecific and interspecific

genetic distance analysis. According to the intraspecific genetic distance analysis, the Javan hawk-eagle (*N. bartelsi*) has no genetic variation within the species with a genetic distance of 0.000 (Table 4). This implied that there was no genetic variation within the species group of *Nisaetus bartelsi* consisting of the five samples; thus, it did not only confirm the species label as Javan Hawk-Eagle (*Nisaetus bartelsi*) but also the fact that the five individuals COI gene sequences were conserved regardless the habitat fragmentation that isolates the population. However, to confirm this, more data on COI gene sequences of Javan hawk-eagle is needed.

Table 3. Pairwise genetic distance amongst *N. bartelsi*, *S. alboniger* (ingroup; accession code: AP008239), and *Sagittarius serpentarius* (outgroup; accession code: U83776).

	RR24	FW01	FM01	RR21	RR04	AP008239	U83776
RR24		0.000	0.000	0.000	0.000	0.007	0.0027
FW01	0.000		0.000	0.000	0.000	0.007	0.027
FM01	0.000	0.000		0.000	0.000	0.007	0.027
RR21	0.000	0.000	0.000		0.000	0.007	0.027
RR04	0.000	0.000	0.000	0.000		0.007	0.027
AP008239	0.021	0.021	0.021	0.021	0.021		0.026
U83776	0.217	0.217	0.217	0.217	0.217	0.209	

Table 4. The intraspecific genetic distance of *N. bartelsi* against few species from Genbank.

Genbank species	Distance	Standard Error
<i>S. tyrannus</i>	n/c	n/c
<i>N. bartelsi</i>	0.000	0.000
<i>S. alboniger</i>	n/c	n/c
<i>N. nipalensis</i>	0.000	0.000
<i>Sagittarius serpentarius</i>	n/c	n/c
<i>Aquila chrysaetos</i>	0.000	0.000
<i>S. melanoleucus</i>	n/c	n/c
<i>N. cirrhatus</i>	n/c	n/c
<i>S. philippensis</i>	n/c	n/c

n/c: not covered and genetic distance's value was very high

Interspecific genetic distance was analyzed to disclose the relationship amongst the species *N. bartelsi* with the other closely related taxa. The data revealed that *Nisaetus alboniger* had a genetic distance of 0.013 compared to *N. bartelsi*, which supports its status as the sister species of the Javan

hawk-eagle (*N. bartelsi*). Moreover, the next closest relative of *N. bartelsi* was *Nisaetus nipalensis*, with a genetic distance of 0.021. The furthest relative of *N. bartelsi*, according to the interspecific genetic distance, was *S. tyrannus*, with a genetic distance of 0.111 (Table 5).

Table 5. Interspecific genetic distance of few genus in Accipitridae family.

C	S.	N.	S.	N.	<i>Sagittarius</i>	<i>Aquila</i>	S.	N.	S.
	<i>tyrannus</i>	<i>bartelsi</i>	<i>alboniger</i>	<i>nipalensis</i>	<i>serpentarius</i>	<i>chrysaetos</i>	<i>melanoleucus</i>	<i>cirrhatius</i>	<i>philippensis</i>
<i>S. tyrannus</i>		0.017	0.016	0.016	0.074	0.016	0.014	0.016	0.015
<i>N. bartelsi</i>	0.111		0.005	0.007	0.071	0.015	0.014	0.013	0.014
<i>S. alboniger</i>	0.108	0.013		0.006	0.071	0.015	0.014	0.013	0.014
<i>N. nipalensis</i>	0.103	0.021	0.021		0.069	0.015	0.014	0.013	0.014
<i>Sagittarius serpentarius</i>	0.870	0.850	0.856	0.850		0.072	0.072	0.071	0.071
<i>Aquila chrysaetos</i>	0.099	0.088	0.088	0.086	0.850		0.015	0.015	0.016
<i>S. melanoleucus</i>	0.076	0.091	0.093	0.088	0.870	0.091		0.014	0.014
<i>N. cirrhatus</i>	0.102	0.081	0.076	0.081	0.850	0.091	0.089		0.009
<i>S. philippensis</i>	0.094	0.089	0.083	0.089	0.863	0.101	0.081	0.037	

Phylogenetic tree of javan hawk-eagle.

According to the neighbor-joining tree reconstruction, we confirmed that the five samples, FM01, FW01, RR04, RR21, and RR24 belonged to the same species since they formed a single clade with a bootstrap value of 97. The tree also confirmed that the reference sequence, *S. alboniger* AP008239.1 and *N. bartelsi* were sister

species, with a bootstrap value of 88. They formed a monophyletic branch along with *N. nipalensis*, with a bootstrap value of 97. This monophyletic group split from the other *Spizaetus* groups, consisting of *S. tyrannus*, *S. melanoleucus*, *N. cirrhatus*, and *S. philippensis* (Figure 2).

Figure 2. Phylogenetic tree of Javan hawk-eagle and the other species in *Accipitridae* family.

The Javan hawk-eagle used to be grouped in the genus *Spizaetus*, which distributes in South America, due to their shared morphological characteristics, including long tails and feathered tarsi. The latest studies at the molecular level suggest that all hawk-eagle species in Asia separated earlier from genus *Spizaetus* and formed a monophyletic branch named *Nisaetus* (Gamauf *et al.*, 2005; Helbig *et al.*, 2005; Lerner *et al.*, 2017). However, the new phylogeny and taxonomy did not necessarily change the registered sequence names in the Genbank of the NCBI database. This explains why several reference sequences in this study still bear the genus name *Spizaetus*.

Genus *Nisaetus* comprises all species of hawk-eagles distributed in Asia, including Southeast Asia, with some species endemic to particular areas (Lerner *et al.*, 2017). The geographical condition of southeast Asia is the major factor for its high rate of species endemism, with the Javan hawk-eagle as an example (Gamauf *et al.*, 2005). The Javan hawk-eagle particularly inhabits the primary rainforests of Java and shares the habitat generically with the changeable hawk-eagle (*N. cirrhatus*) (Nijman, 2004). The two species prefer similar prey. Thus, they also share the same ecological role as top-order predators. Despite the similarities in their ecological roles on the island of Java, according to this study, the two species are not closely related; in fact, the two species sit on different clades.

On the other hand, according to this study, the Javan hawk-eagle was at the same clade with the Blyth's hawk-eagle (*N. alboniger* or *S. alboniger* AP008239.1), which is distributed in the closed forests of Sumatra and Borneo, as sister species. Morphologically, the Javan hawk-eagle shares many characteristics with the Blyth's hawk-eagle (*N. alboniger*), such as the feathered tarsi, plumage pattern and distinct crest, other than their color. *N. bartelsi* has cinnamon brown plumage that grows darker, barred breast and stripes that grow clearer as the bird ages. Meanwhile, its sister species, the Blyth's hawk-eagle (*N. alboniger*), has black and white plumage with a spotted breast and barred underparts (IUCN, 2016). This is because of the Javan hawk-eagle (*N. bartelsi*) along with the Blyth's hawk-eagle (*N. alboniger*) came from different lineages of the changeable hawk-eagle (*N. cirrhatus*) (Gjershaug, 2006).

The changeable hawk-eagle (*N. cirrhatus*) has a much wider distribution, ranging throughout southeast Asia, specializing in forest edges and open woodlands (Ferguson & Christie, 2001). In Java, it can also be found in primary rainforests, along with the Javan hawk-eagle (Nijman, 2004). However, the interspecific genetic distance data reveals that the two species are not closely related, despite their similar habitat preferences and hunting behaviors. The high interspecific genetic distance between *N. bartelsi* and *N. cirrhatus* (0.081) indicates that the two groups had already split even before the South American and African hawk-eagle lineages separated (Lerner & Mindell, 2005; Haring *et al.*, 2007); a close interspecific genetic distance of 0.013 with *N. alboniger* shows that the Javan hawk-eagle (*N. bartelsi*) split from their ancestral population due to the rising sea level, which separated Java from Sumatra and Borneo and isolated the Javan hawk-eagle population (Gamauf *et al.*, 2005; Haring *et al.*, 2007; Lerner *et al.*, 2017).

Moreover, the Javan hawk-eagles and the changeable hawk-eagles in Java are segregated by habitat, by the topography of the terrains, and partially by altitude. *N. bartelsi* occurs over a smaller range of habitats than *N. cirrhatus*. The once dense continuous rainforest of Java has been fragmented into numerous small patches or has been replaced by more open habitat types, to which Javan hawk-eagles are not well adapted (Nijman, 2004). This condition enhances the significant genetic divergence with the changeable hawk-eagle and generates this species' endemism as it prevents gene flow from one population of Javan hawk-eagles to the others (Gamauf *et al.*, 2005).

A reliable diagnosis of the taxonomic status of populations is essential for conservation because unrecognized species may become extinct due to a lack of information, including genetic information, which can lead to a lack of protection (Gamauf *et al.*, 2005). A taxonomic unit as detailed as the species level is usually prioritized in conservation measures as an evolutionarily significant unit (Frankham, 2002). As a result, the phylogenetic species concept affects conservation more than any other disciplines (Collar, 1997).

CONCLUSION

The COI gene sequences obtained from the five samples with the code names FM01, FW01, RR04, RR21, and RR24 had 744, 742, 734, 724, and 741 base pairs, respectively.

According to the phylogenetic analysis, Javan hawk-eagles FM01, FW01, RR04, RR21, and RR224 was confirmed as one species, *N. bartelsi*, a sister species of *Nisaetus (Spizaetus) alboniger* AP008239.1. Thus, the DNA barcoding of the Javan hawk-eagle conducted in this study is a stepping stone to conservation efforts for the Javan hawk-eagle.

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