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CO2 Mitochondrial Gene Identification of *Nisaetus cirrhatus* as a Part of Indonesian Elang Brontok Genetic Conservation

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ABSTRACT
The Elang Brontok, scientifically named as *Nisaetus cirrhatus* is included in the least Concern in appendix II. The use of the *COI* gene for taxonomic in our previous study put our samples in a complex species taxonomical position. Further research needs to be done using other mitochondrial genes such as *CO2*. In addition, the *CO2* sequence of *Nisaetus cirrhatus* has not yet been reported. The purpose of this study was to obtain complete sequences of *COI* and *CO2* and determine the taxonomic position of the Elang Brontok (*Nisaetus cirrhatus*) in Indonesia. In this study, we used the same three samples as of the previous study for CO1. We designed a pair of primers based on the previously found CO1 sequence with not yet known the length of the target gene since there are no reports for it. Phylogenetic tree reconstruction proved that the Elang Brontok we studied belongs to *Nisaetus cirrhatus*. The result of combining sequences obtained from this study with one previously reported showed some mutations at the 5' end of *COI*. We suggest that the extended 3' end of *COI* is part of the yet reported *Nisaetus cirrhatus CO2* sequence.

Keywords: *Genetic conservation, Indonesia, MT-COI, MT-CO2, Nisaetus cirrhatus.*

1. INTRODUCTION

The Elang Brontok (*Nisaetus cirrhatus*) is one of the predator species which is included in the *Least Concern* category regarding their latest existence [1]. It is predicted to quickly enter the endangered phase based on the current fact that the adult number recorded is less than 10,000 [1]. Illegal hunting and trading, deforestation, and the narrowing of its natural habitat are blamed to be the cause of this decline [1], [2]. Researchers have long predicted that the eagle population will continue to decline if there is no serious efforts are made to protect the ecosystem [3]–[5].

Inbreeding is another problem highly concerned with the management and conservation of this species[6]–[8]. This behavior along with genetic drift has worsened its impact on decreasing the ability to survive [4], [8], [9]. One of the conservation strategies applied out in Indonesia to protect the *Nisaetus cirrhatus* species from further population decline is to obtain genetic information as a basis for releasing actions to suit their natural habitat [7], [10]–[12]. This effort includes the genetic detection of possible inbreeding among individuals using their genomic data. *Nisaetus cirrhatus* passes three phases of life which morphologically are characterized by their feather color transition [13]. This color change is also influenced by seasons. Concerning that special character of *Nisaetus cirrhatus* described above, genetic identification is necessary to support the phenotypic identification technique which is susceptible to species complex and cryptic species. Genetically identification helps to clarify the taxonomic position of phenotypically resembles individuals which often classify them whether in species complex or even cryptic species [13]–[15].

Table 1. The similarity of Nisaetus cirrhatus with Nisaetus nipalensis and Nisaetus alboniger

Sample	Query Cover	Similarity	Species	Accession No.
RR14	98 %	93,77 %	*Nisaetus nipalensis*	(AP008238.1)
RR15	100 %	93,62 %		
RR16	99 %	93,89 %		
RR14	99 %	93,35 %	*Nisaetus alboniger*	(AP008239.1)
RR15	100 %	93,49 %		
RR16	99 %	93,64 %		

In this last decade DNA Barcode is widely used to identify unknown, disputed species or even the original habitat of captured or illegally domesticated animals [13], [16]–[19]. By using short sequences of mitochondrial genes this technique is very well approved of its capability in detecting a significant genetic differentiation through the reconstructed phylogenetic tree [5], [13], [20]. *Control region* (CR) [21], *COI* [16], [17], [22], and *D-loop* [23] genes are those among mitochondrial genes which have been widely used for animal identification. The *COI* gene is a mitochondrial gene that encodes Cytochrome Oxidase subunits-1 which is slightly mutated [17] and classified as one of the conserved genes among organisms. The downstream of CO1 is *CO2* which is interrupted with one or two t-RNA sequences [24]. Previous studies reported the identification of *Nisaetus cirrhatus* based on the ND3 and ND4L (Mandasari & Listyorini, *Data Set*) genes but failed to determine the position of this bird's taxon to the species level. Another study using the *COI* [25] gene found a species complex in *Nisaetus cirrhatus*. It was revealed that the *COI* gene fragment amplified in that study has not yet reached its 3' end. Furthermore, identification of *CO2* and other mitochondrial genes is believed to help a more accurate identification of this eagle when whole mitochondrial genome identification is not possible to be conducted due to some reasons. This research was a part of the Raptor Mitochondrial Genome Project with De Novo approach which is aimed to identify raptors mitochondrial genes, genetic conservation, providing genetic information database to the GenBank and BOLD Systems, especially eagles living in Indonesia, including Elang Brontok (*Nisaetus cirrhatus*). More specifically, this study aims to obtain the complete *COI-CO2* sequence of *Nisaetus cirrhatus* and to know the taxonomic position of this bird more precisely. In this research 3' end of *COI* was retrieved along with *CO2* fragment to complete our previous work.

2. METHODOLOGY

This research was conducted from August 2020 – to July 2021. Blood samples from Elang Brontok (*Nisaetus cirrhatus*) were collected from Cikananga Wildlife Center, Sukabumi. In this study, three samples among others encoded as RR14, RR15, and RR16 were analyzed. Total DNA isolation was conducted following the procedure provided by the manufacturer of QIAmp® DNA Mini (Qiagen Cat. No. 51304) in Spin Protocol. Total DNA quantity and quality were measured on NanoDrop ND-2000 Spectrophotometers (ThermoScientificTM). Amplification of the 3'CO1 end sequence gene by PCR technique using Qiagen Dream Taq Green PCR Master Mix in RotorGene Q® machine and a pair of primers as follow: forward primer 3'*COI*-F 5'-CTC TTC TGG TTC TTC GGA CA-3' and reverse primer 3'*COI*-R 5'-GCA GCC GTG GAT TCA TTC A-3'. Amplification was done in 40 cycles with pre-denaturation at 95^{o}C for 3 minutes, cycles of denaturation at 95^{o}C for 1 minute, annealing at 52^{o}C for 1 minute, and extension at 72^{o}C for 1 minute, and then final extension at 72^{o}C for 10 minutes. The amplified fragment integrity was examined using the electrophoresis technique in 0.8% agarose gel for 60 minutes at 50 volts, then visualized on a UV transilluminator. Appropriate samples were sent to First Base Laboratories, Malaysia for sequencing. The sequencing was read using Bioedit application which was then analyzed in DNA Baser application to obtain a consensus sequence from forward and reverse fragments. To determine the target gene fragments obtained, BLAST (*Basic Local Alignment Search Tool*) analysis was carried out. Multiple alignments were done using MEGAX to more precisely identify the fragment. Phylogenetic tree reconstruction was done to clarify the taxonomic position using a longer sequence than reported before in *Minimum Evolution (ME), Maximum Likelihood (ML), Neighbor-Joining (NJ),* and *Maximum Parsimony (MP)* methods with *Kimura-2 parameters* and 1000 bootstraps.

Table 2. Length differences and mutations of the samples compared to *Nisaetus cirrhatus* (ROMC331-07)

Sample	5' end sequence		Nucleotides				3' end excess
	lack	differences	39	87	330	477	
RR14	12 bp	7 bp	-	-	C > T	-	757 bp
RR15	21 bp	4 bp	-	G > A	C > T	C > T	742 bp
RR16	22 bp	3 bp	C > T	G > A	C > T	C > T	758 bp

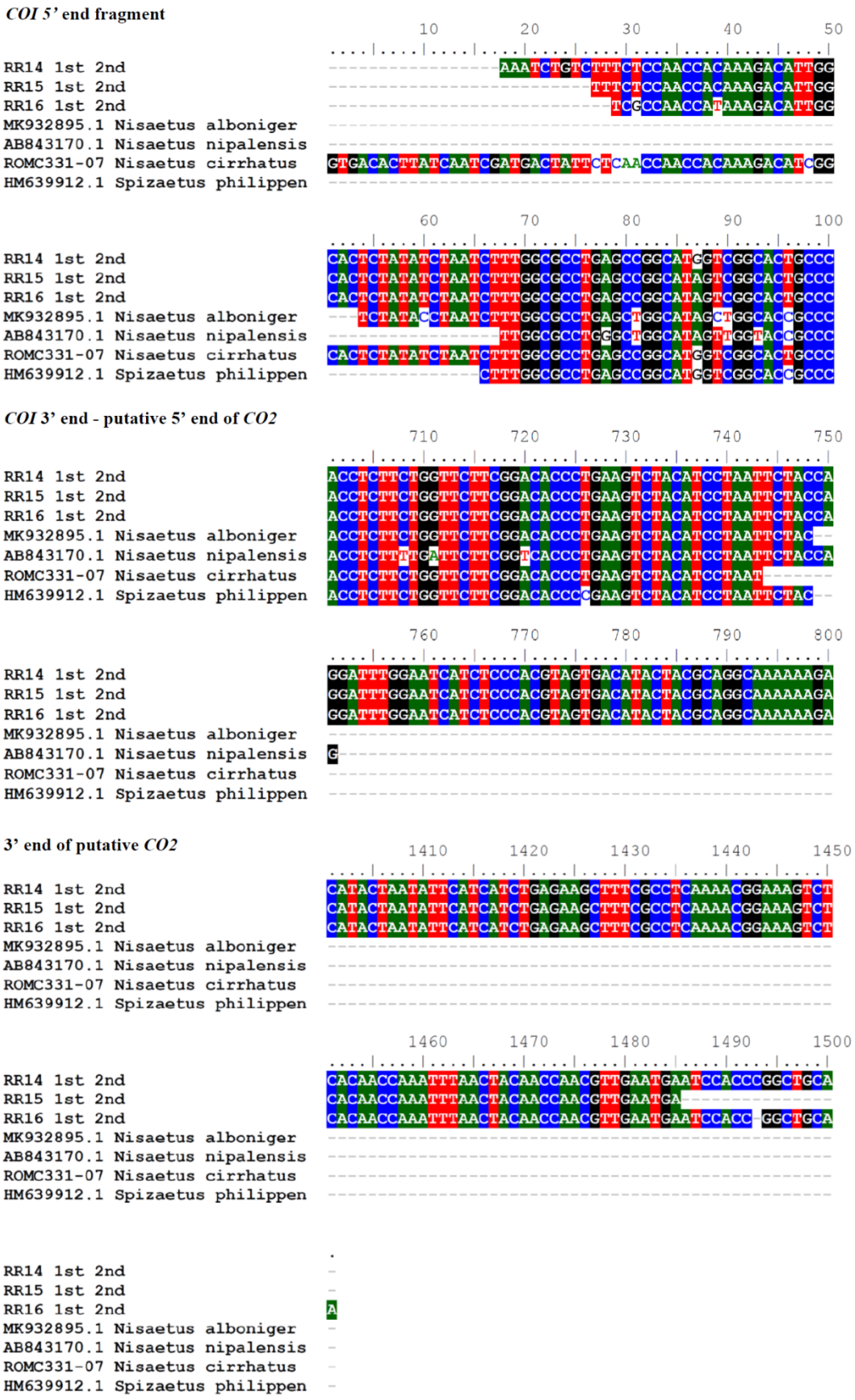

Figure 1. Multiple Alignment of combined RR14, RR15, and RR16 fragments.

Table 3. Pairwise Distance between studied samples

		1	2	3	4	5	6	7
1	RR16 1st 2nd		0.0000000	0.0021208460	0.0024667010	0.0109800274	0.0076986170	0.0123328624
2	RR15 1st 2nd	0.0000000		0.0021208460	0.0024667010	0.0109800274	0.0076986170	0.0123328624
3	RR14 1st 2nd	0.0029673678	0.0029673678		0.0014130419	0.0111954834	0.0073049268	0.0124048211
4	ROMC331-07 Nisaetus cirrhatus COI-5P	0.0044576818	0.0044576818	0.0014814826		0.0111652946	0.0075603915	0.012284947
5	MK932895.1 Nisaetus alboniger voucher KU583 COI	0.0794470110	0.0794470110	0.0794470110	0.0777287962		0.0117307727	0.0068014288
6	HM6339912.1 Spizaetus philippensis voucher PEF32 COI	0.0367203122	0.0367203122	0.0335557447	0.0351355248	0.0863795310		0.0128985764
7	AB843170.1 Nisaetus nipalensis isolate: BJNSM710-11 COI	0.0934095238	0.0934095238	0.0934095238	0.0916427375	0.0335557447	0.1005397694	

3. RESULTS AND DISCUSSION

Isolation of genomic DNA produced pure total DNA. The consensus sequence of the target gene from three samples RR14, RR15, and RR16 were 798 bp, 784 bp and, 790 bp, respectively. BLAST analysis revealed the high query coverage (98%-100%) with similarity 93,63% up to 93,89% compared to *Nisaetus nipalensis* complete genome (AP008238.1) and 93.35% up to 93.64% compared to *Nisaetus alboniger* complete genome (AP008239.1) (Table 1). It is confirmed that the fragments obtained were fragments of the targeted gene.

Combining our finding with the previously reported fragment [25] resulted in 1,465 bp (RR14), 1,453 bp (RR15), and 1,453 bp (RR16) fragments, respectively. Multiple alignments of those combined fragments with *Spizaetus philippensis* (HM639912.1), *Nisaetus alboniger* (MK932895), *Nisaetus nipalensis* (AB843170.1), and *Nisaetus cirrhatus* (ROMC331-07) as main references revealed the differences in fragment length and some mutations in those three samples. Sample RR14 was 12 bp, while RR15 and RR16 were 21 bp and 22 bp shorter in their *COI* 5' end fragment compared to *Nisaetus cirrhatus* (ROMC331-07) at the 5'*COI*. Meanwhile, those three samples showed 757bp, 742bp, and 758bp excess beyond the 3' end of *COI* sequence, respectively. The comparison against *COI* of *Nisaetus alboniger* (MK932895.1) showed an excess of 36 bp, 27 bp, and 25 bp at the 5' end and an excess of 747 bp, 737 bp, and 753 bp at the 3' end, respectively; and comparation against *COI* of *Nisaetus nipalensis* (AB843170.1) showed an excess of 50 bp, 43 bp, and 39 bp at the 5' end and an excess of 752 bp, 737 bp, and 753 bp at the 3' end, respectively (Table 2; Figure 1).

Pairwise Distance analysis of obtained fragments compared to the different references in the same genus, including *Spizaetus philippensis* (HM639912.1), *Nisaetus alboniger* (MK932895), *Nisaetus nipalensis* (AB843170.1), and *Nisaetus cirrhatus* (ROMC331-07) showed that the genetic distances of those three samples were 0.04, 0.02, and 0.00, respectively (Table 2). It confirmed that studied samples belong to the species *Nisaetus cirrhatus* (ROMC331-07). This finding had clarified the species complex claim as reported previously which is also supported by phylogenetic tree reconstruction results [25]. In general, all four phylogenetic tree reconstruction methods elaborated in this study using combined *COI-CO2* fragments gave the same result; that all three samples grouped in the same clade with the main reference *Nisaetus cirrhatus*. More careful observation revealed that small differences are found among those three samples, yet it is consistent in all methods (Figure 2-5). The RR14 sample was closer to *Nisaetus cirrhatus* compared to RR15 and RR16. This confirms the finding that the eagle genus is still evolving, while RR15 and RR16 bear a genetic distance from RR14 and *Nisaetus cirrhatus*. We suggest that this fact is a result of some mutations and different fragment lengths successfully amplified in this study. All analysis also confirmed that there is no subspecies formation suggested to be or ongoing to be formed. Combining samples' fragment sequences of RR14, RR15, and RR16 obtained in this study with the previously reported sequence resulted in a longer fragment than the *COI* sequence compared to the references including *Spizaetus philippensis* (HM639912.1), *Nisaetus alboniger*

(MK932895), *Nisaetus nipalensis* (AB843170.1) available in NCBI and *Nisaetus cirrhatus* (ROMC331-07) available in BoldSystem. We suggest that the sequences obtained covered the *CO2* sequence, which so far there has been no specific report for all types of raptors. The assumption that the excess fragment is *CO2* of the *Nisaetus cirrhatus* species is based on the mitochondrial genome-map of the *Aquila fasciata* species, which is one of the raptors closely related to *Nisaetus cirrhatus*. We also suggest that there is sequence/s of t-RNA which are normally located adjacent to the *COI* and connect it with *CO2*.

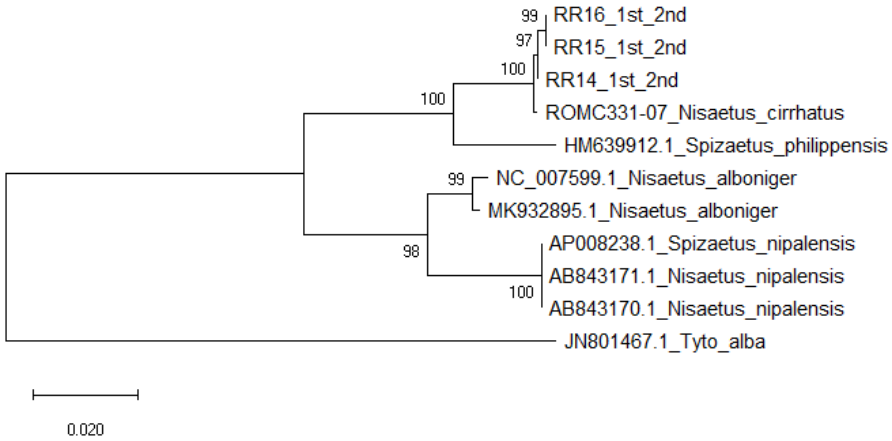

Figure 2. Phylogenic tree topology based on *Neighbor-Joining* (*NJ*) of combined RR14, RR15, and RR16

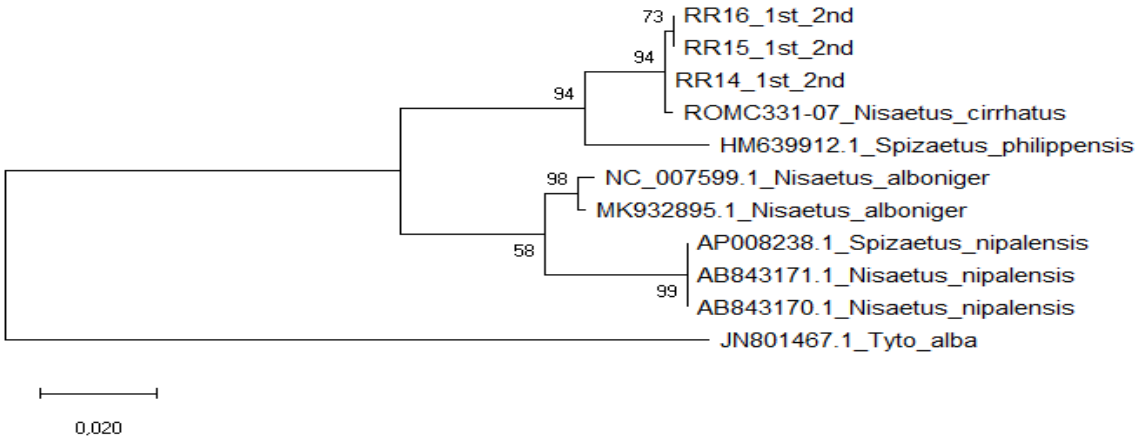

Figure 3. Phylogenic tree topology based on *Maximum Likelihood (ML)* of combined RR14, RR15, and RR16 samples

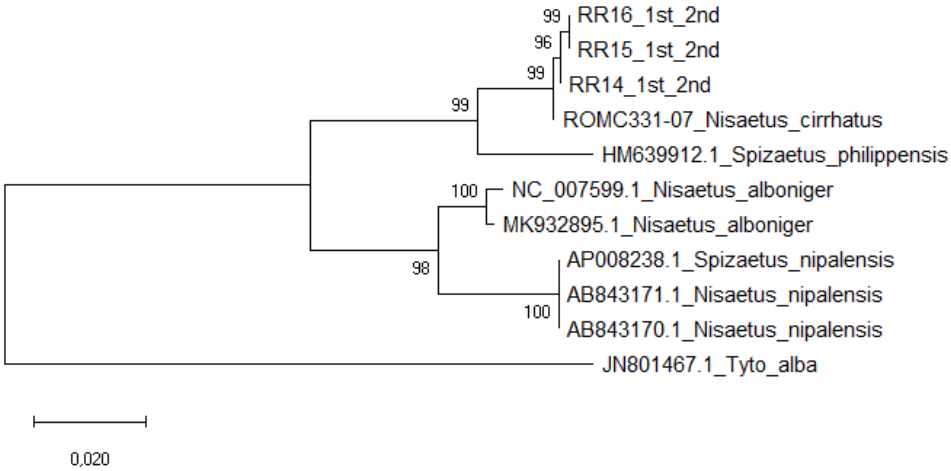

Figure 4. Phylogenic tree topology based on *Minimum Evolution (ME)* of combined RR14, RR15, and RR16 samples

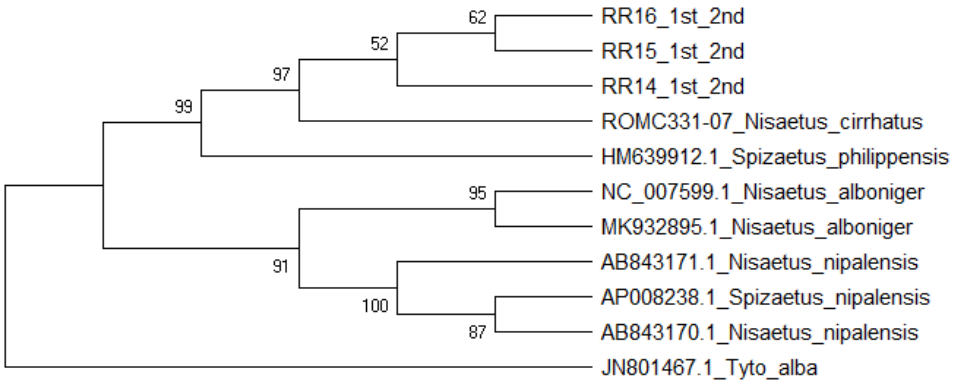

Figure 5. Phylogenic tree topology based on Maximum Parsimony (MP) of combined RR14, RR15, dan RR16 samples

The consistency of the position of RR14, RR15, and RR16 samples acquired from the phylogenetic topology analysis, apart from mutations found in every sample, is thought to be caused by the wide distribution of *Nisaetus cirrhatus* which may result in genetic diversity in the population [1]. The wide distribution of *Nisaetus cirrhatus* also causes monophyletic formation with other species [14], [24]. This is evidenced by the discovery of subspecies of *Nisaetus cirrhatus* in the Asian region which includes *atus* cirrhatus (India), *Nisaetus cirrhatus* ceylanensis (Sri Lanka), *Nisaetus cirrhatus* andamanensis (Andaman Islands), *Nisaetus cirrhatus* limnaeetus (Bangladesh, Myanmar, and Nepal. Indochina and the Malay Peninsula and Southeastern Philippines), and *Nisaetus cirrhatus* vanheurni (Sumatra) [14], [26]. Further analysis is required to be able to depict the anatomy of the *COI-CO2* sequence, especially for Elang Brontok living in Indonesia. Since there is no report on *CO2* sequence neither in GenBank of NCBI nor in BOLD System. It is necessary to soon submit this finding to get confirmation or a discussion with researchers working on raptors mitochondrial genome or any Avian ones.

This study succeeded in completing the previously reported 3' of Elang Brontok *COI* sequence rehabilitated in Cikananga Wildlife Center. The excess fragments obtained in this study are suggested to be a *CO2* sequence or part of *CO2* with t-RNA/s located in-between. The De Novo approach elaborated will surely take an extended time to retrieve a complete sequence of this raptor mitochondrial DNA. We are still trying to build a research collaboration that may support the whole mitochondrial genome analysis in one using an advanced sequencing technology such as NGS or Nanopore sequencing technology.

AUTHORS' CONTRIBUTIONS

The research is part of D.L's research project. The conceptualization of this project was mostly completed by D.L. and R.K. Methodology development by R.K and D.L., data analysis by R.K and R.L.K; S.K.H.I., and A.S prepared the figures; R.K and D.L prepared and wrote the original draft; D.W.P., D.N., and R.L.K. carried out a massive critical revision of the manuscript; supervision was conducted by D.L; All authors have read and agreed to the published version of the manuscript.

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