GENETIC VARIABILITY COI GENE OF WREATHED HORNBILL IN TAMAN MINI INDONESIA INDAH, INDONESIA*)

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ABSTRACT

The survival of Wreathed Hornbill (*Rhyticeros undulatus*) in Indonesia is threatened due to deforestation as well as hunting for pets. We can find these birds in Sumatra, Borneo, and Java. Ex-situ conservation through zoo or bird park provides an alternative for species preservation from its extinction. However, often the origin population of the species is unknown. Thus, the study was aimed to verify the species, genetic variability, and single nucleotide polymorphism (SNP) of the Wreathed Hornbill collection Taman Mini Indonesia Indah (TMII) based on COI gene. Tissue sample was collected from blood and DNA extraction was conducted using Dneasy® Blood and Tissue Kit, and PCR amplification using Primer COIBuceF and COIBuceR. We used BLAST method to verify the species by comparing with nucleotide sequences in GenBank. Genetic distance calculated using the Neighbor-Joining method with Kimura 2-parameter models. We found from seven nucleotide sequences indicated that the percentages of the species identity was 94% with *Aceros corrugatus* (GenBank: HM755883). There are five haplotypes of Wreathed Hornbill in TMII i.e. first haplotype is RU1TM, second haplotype RU2TM, third haplotype RU3TM, fourth haplotype RU6TM, and fifth haplotype RU4TM, RU5TM, and RU7TM. The average of genetic distance between individuals 0.23%. Specific SNP between the haplotypes found in 115 (T115C), 147 (T147C), 369 (T369A), and 372 (T372A) nucleotide sequence. TMII Wreathed Hornbill consists of five haplotypes probably derived from different islands in Indonesia.

Keywords: *species identity, genetic variability, Wreathed Hornbill,* Rhyticeros undulatus, *COI gene.*

INTRODUCTION

Rhyticeros undulatus (Wreathed Hornbill) is one of the Rhyticeros genera, family of Bucerotidae found in Indonesia. Another name of this species is *Aceros undulatus,* distributed over Sumatra, Borneo, and Java (Sukmantoro et al., 2007; MacKinnon et al., 2010). Its global distribution are India, Bangladesh, Bhutan, Brunei Darussalam, Cambodia, Indonesia, Laos, Malaysia, Myanmar, Thailand, and Vietnam (Krishna et al., 2012).

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This bird has an important role in the forest. One of its roles is as a plant seed dispenser (Kinnaird, 1998; Kitamura et al., 2008; Balasubramanian et al., 2011). Wreathed Hornbill is more of a generalist frugivore when compared with the other species of hornbills such as the Great Pied and Brown Hornbill (Datta dan Rawat, 2003). Hornbills feed mostly on figs (Poonswad, 2016).

Unfortunately, the existence of Wreathed Hornbill in Indonesia forest being threatened. The main factor causing the threat is deforestation. Hornbills are highly sensitive to habitat fragmentation due to deforestation (Kinnaird and O'Brien, 2007). Impacts of deforestation on hornbills are habitat, food sources, and nesting trees losses (Kinnaird, 1998; Kitamura et al., 2008; Balasubramanian et al., 2011). In addition, illegal trade also contributes significantly to the acceleration of the extinction rate of Wreathed Hornbill. Wreathed Hornbill is traded illegally in Indonesian traditional markets. Generally, birds that are traded captured from wild habitats. Law enforcement officials often get of Hornbill including Wreathed Hornbill from illegal traders.

Generally, this Wreathed Hornbill was submitted to zoo. But the history of its origin is frequently unclear. In the zoo this birds was often mated to couples who's its origins also unclear. Unclearly of Wreathed Hornbill origin that mated in Indonesia zoos, including Taman Mini Indonesia Indah can increase the risk of inbreeding. High inbreeding will threaten the survival of the population, because it will produce offspring that have low survival, less weight, and sterile (Selander, 1983; Ralls et al., 1988; Haig and Nordstrom, 1991).

This study aims to describe the genetic variability of Wreathed Hornbill in Taman Mini Indonesia Indah using cytochrome oxidase unit I (COI) mitochondrial DNA. The number of haplotypes obtained can illustrate the relationship between populations and its geographic origin.

MATERIALS AND METHODS

The research was conducted from June to November 2016. The Wreathed hornbills were sampled in Taman Mini Indonesia Indah (TMII), Jakarta Indonesia. We got seven of Wreathed hornbill in this place. Molecular analysis carried out in the Laboratory of Molecular Biology, Research Center for Biological Resources and Biotechnology (PPSHB), Bogor Agricultural University (IPB).

The DNA sample yielded through the Wreathed hornbill blood. Blood is taken out from the ulnar vein as much as 0.1-0.5 ml, and then put into a 1.5 ml effendorf tube which

already contains EDTA (Seutin *et al.*, 1991). Each bottle of blood samples is separately labeled.

Total DNA isolation follows Spin-Column Protocol, using DNeasy Tissue Kit ®Blood and paint No. 69 504 (50). Approximately 25 mg samples of blood inserted into the effendorf tube accordingly, before added with 200 µl ATL buffer and crushed. Added 20 µl Proteinase-K, vortexes for 3 minutes. Samples then incubated at 56° C for ± 2 hours, regularly vortexes in every 20 minutes. A total of 200 µl of AL buffer added to the tubes, vortexes and incubated at 56° C for 10 minutes. Added 200 µl absolute ethanol, and stored in freezer for 2 hours. Supernatant formed then transferred to spin column and centrifuge 8000 rpm for 1 minute. Remove the solution in the reservoir tube. Add 500 µl AW1 solution (wash buffer), centrifuged 8000 rpm for 1 minute, remove the liquid in the container. Add 500 µl AW2 solution (wash buffer), centrifuged in 13000 rpm 3 minutes and repeat for 1 minute. Tubes spin column was transferred to effendorf, add 50 µl solutions of AE (elution buffer) and let stand 30 minutes, centrifuge 8000 rpm 1 minute, and keep the DNA into the freezer.

We used polymerase chain reaction (PCR) technic to identify differences of COI gene nucleotide sequences. Primer was designed with Primer3 (http://bio-info.ut.ee/primer3-0.4.0/primer3) software based on aligning sequences of *Aceros waldeni* (GenBank: NC-015085). Our primer name is COIBuceF and COIBuceR with 750 bp of product size.

Composition of running buffer were consisted of 2 µl DNA template, 1.0 µl of forward and reverse primer (20 pmol/µl), 6.8 µl ddH2O, 5,0 µl Qs buffer, 5.0 µl of Enhancer, 1.0 µl of dNTP, and 0.2 µl Taq polymerase. The PCR conditions were 95°C of pre-denaturation (5 min), 94°C of denaturation (1 min), annealing with 54°C (45 sec), and extension at 72°C temperature (1 min). DNA amplification products were migrated in 1.2% agars gel (Sambrook, 1989). We used of First BASE laboratories in Malaysia to sequencing our PCR product.

Nucleotides sequences (forward and reverse) from First BASE were edited and aligned using Clustal W methods with MEGA 6.0 software. We were constructed of phylogenetic tree using Neighbor-Joining models with 1000 repetitions bootstrap. Genetic distances analysis based on Kimura 2-parameters (Tamura *et al.*, 2011).

RESULTS

Characterization of the Mitochondrial DNA COI gene

We analyzed 7 individuals of Wreathed Hornbill in this study. The length of Wreathed Hornbill COI genes that can be amplified by PCR machine were 750 bp (Fig. 1). From this length, we got 746 bp of COI gene sequence.

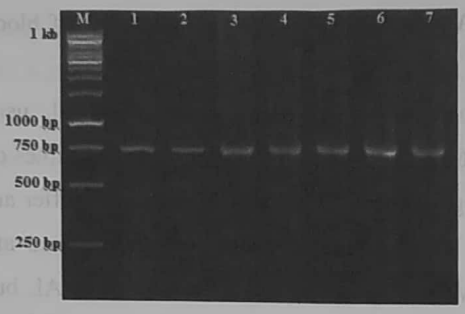

Figure 1. Documentation of DNA band mtDNA COI gene which amplified by PCR machine. Description: 1 = RU1TM, 2 = RU2TM, 3 = RU3TM, 4 = RU4TM, 5 = RU5TM, 6 = RU6TM, 7 = RU7TM. RU = Rhyticeros undulatus, 1-7 = individual sequence, TM = Taman Mini Indonesia Indah.

Multiple alignment results of seven Wreathed Hornbill mtDNA COI genes showed conservative nucleotides as much as 99.32% (Table 1). Nucleotide sites variable 6.70%, and singleton sites was only 5.36%. The nucleotide base composition of COI gene sequence were Cytosine (C) 33,9%, Adenine (A) 24,8%, Thymine (T) 23,9%, and Guanine (G) 17,4%. The composition of GC were 51.3% and AT 48.7%. The BLAST result shows that the identities of all individuals were 94% (Table 2) and 100% query cover.

Table 1. Nucleotide sequences characterization of Wreathed Hornbill COI genes in 746 bp length

No	Character	Rhyticeros undulatus (semua individu)
1	Number of individuals	7
2	Nucleotide base length	746
3	Conserved sites (%)	741 (99,32)
4	Variable sites (%)	5 (6,70)
5	Singleton sites (%)	4 (5,36)
6	Percentage of Thymine (T)	23,9
7	Percentage of Cytosine (C)	33,9
8	Percentage of Adenosine (A)	24,8
9	Percentage of Guanine (G)	17,4

Table 2. BLAST (*Basic Local Alignment Search Tool*) result of Wreathed Hornbill COI gene with 746 bp length with GenBank

No	Sample	Total Score	Query Cover (%)	Identity (%)	Species	GenBank Accession Number
1	RU1TM	1123	100	94	*Aceros corrugatus*	HM755883.1
2	RU2TM	1118	100	94	*Aceros corrugatus*	HM755883.1
3	RU3TM	1123	100	94	*Aceros corrugatus*	HM755883.1
4	RU4TM	1123	100	94	*Aceros corrugatus*	HM755883.1
5	RU5TM	1123	100	94	*Aceros corrugatus*	HM755883.1
6	RU6TM	1129	100	94	*Aceros corrugatus*	HM755883.1
7	RU7TM	1123	100	94	*Aceros corrugatus*	HM755883.1

Single Nucleotide Polymorphism and Genetic Distance

We found four site of single nucleotide polymorphism (SNP) as the differentiation between Wreathed Hornbill individuals (Table 3). These SNP sites were located in 115 (T115C), 147 (T147C), 369 (T369A), and 372 (T372A) of the 746 bp nucleotide sequences. The nucleotide bases that distinguish the four sites were T.

The average genetic distance between individuals *Rhyticeros undulatus* was 0.23% (Table 4). Genetic distance of Wreathed Hornbill group with *Rhyticeros plicatus* (Blyth's Hornbill) was 1.63%, Wreathed Hornbill group and *Aceros cassidix* (Knobbed Hornbill) 4.35%, and Wreathed Hornbill group and *Aceros waldeni* 6.63%. Furthermore, the genetic distance between the first haplotype and others is 0.27%, second haplotype with others 0.31%, third haplotype with others 0.26%, and fifth haplotype with others 0.26%.

Table 3. Single Nucleotide Polymorphism (SNP) of COI gene (746 bp) between *Rhyticeros undulatus* individuals

No	Sample	Position of Single Nucleotide Polymorphism			
		115	147	369	372
1	RU1TM	C	T	A	G
2	RU2TM	C	C	T	T
3	RU3TM	T	C	A	G
4	RU4TM	C	C	A	G
5	RU5TM	C	C	A	G
6	RU6TM	C	C	A	G
7	RU7TM	C	C	A	G

Table 4. Genetic distance between individuals of *Rhyticeros undulatus* based on COI gene nucleotide sequence mtDNA (746 bp)

No	Species	1	2	3	4	5	6	7	8	9	10	11
1	RU1TM											
2	RU2TM	0.0040										
3	RU3TM	0.0027	0.0040									
4	RU4TM	0.0027	0.0040	0.0027								
5	RU5TM	0.0027	0.0040	0.0027	0.0000							
6	RU6TM	0.0013	0.0027	0.0013	0.0013	0.0013						
7	RU7TM	0.0027	0.0040	0.0027	0.0000	0.0000	0.0013					
8	RP1TM	0.0163	0.0177	0.0163	0.0163	0.0163	0.0149	0.0163				
9	RP1RG	0.0164	0.0177	0.0164	0.0163	0.0163	0.0150	0.0163	0.0027			
10	AC1RG	0.0219	0.0233	0.2190	0.0219	0.0219	0.0205	0.0219	0.0219	0.0191		
11	*Aceros waldeni* (GB: NC015085)	0.0655	0.0669	0.0655	0.0655	0.0655	0.0640	0.0655	0.0700	0.0701	0.00640	

Notes: RP1TM=*Rhyticeros plicatus*, RG=Taman Margasatwa Ragunan

Relationship and haplotype

Based on phylogenetic trees reconstructed using the Neighbor-Joining method in the MEGA 6 program, there were four main groups of all samples analyzed. Wreathed hornbill clustered each other individual (group I) that closely with group II (*Rhyticeros plicatus*) and group III (*Aceros cassidix*). Separate away with group IV (*Aceros waldeni*) from GenBank (Accession Number: NC-015085).

Group I (*Rhyticeros undulatus*) is divided into five haplotypes. The first haplotype is RU1TM, second haplotype is RU2TM, third haplotype is RU3TM, fourth haplotype is RU6TM, and fifth haplotype is RU4TM, RU5TM, dan RU7TM.

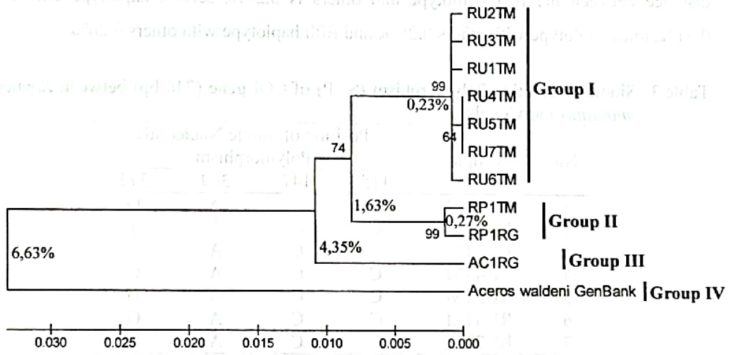

Figure 2. Reconstruction of *Rhyticeros undulatus* phylogenetic tree based on nucleotide sequence of 746 bp partial COI gene using Kimura 2-parameters model and Neighbor-Joining method with 1000x bootstraps.

DISCUSSION

The length of the mitochondrial DNA COI gene found was longer than most of the previously reported. The nucleotide sequence length of the mitochondrial DNA COI gene as a genetic marker for DNA barcode is 648 bp (Hebert et al., 2003).

The BLAST results showed that *R. undulatus* different with *Aceros corrugatus* whose its COI gene nucleotide sequence available in GenBank. The COI gene nucleotide sequence of the *Rhyticeros undulatus* was not found in GenBank.

Based on mtDNA COI gene there were five haplotypes of *Rhyticeros undulatus* in Taman Mini Indonesia Indah. Group I (*Rhyticeros undulatus*) was divided into five haplotypes. The first haplotype is RU1TM, second haplotype is RU2TM, third haplotype is

RU3TM, fourth haplotype is RU6TM, and fifth haplotype is RU4TM, RU5TM, dan RU7TM. It is expected that these five haplotypes will represent their geographical origin. However, this result needs to be tested using others genetic markers of mitochondrial DNA such as COI, Cytochrome-b, and D-loop using DNA source from wild habitat and different geographic. These markers could be using for explain distinguish of Wreathed hornbill population in TMII. Nevertheless, the COI gene (648 bp) of mitochondrial DNA can be used as a DNA barcode for identification of animal species (Hebert, et al., 2004). DNA barcode based on a 650 bp of mitochondrial DNA COI gene is very useful for identifying various animal groups (Hajibabaei et al., 2006).

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