HAYATI
Journal of Biosciences

Vol. 25 No. 4, October 2018 178-187
DOI:10.4308/hjb.25.4.178
ISSN: 1978-3019
EISSN: 2086-4094

DNA Barcode of Seven Indonesian Hornbills Species (Aves: Bucerotidae) Based on Mitochondrial DNA Cytochrome Oxidase Subunit I

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ARTICLE INFO

Article history:
Received June 7, 2018
Received in revised form September 15, 2018
Accepted September 30, 2018

KEYWORDS:
hornbills,
COI gene,
DNA barcode,
genetic distance,
phylogenetic

ABSTRACT

DNA barcoding based on mitochondrial DNA COI gene is very useful in identifying of Indonesian hornbill. We sequenced the DNA barcode of seven hornbill species using the mitochondrial DNA COI gene to explore their genetic variation, identity, distance, and phylogenetic. Thirty-one blood samples from seven hornbill species were isolated and analyzed. Slight variation was observed within the nucleotide of the hornbill species. In contrary, fairly significant difference was shown within the genus and family level. COI gene sequences generated from this study, showed unmatched result with BoLD System database. These seven Indonesian hornbill species were then divided into two groups, namely Group I consisting of *Aceros cassidix*, *Rhyticeros plicatus*, *R. undulatus*, *Buceros rhinoceros*, and *B. bicornis*, while Group II occupied by *Anthracoceros albirostris* and *A. malayanus*; both groups with genetic distance 5.90%. Overall in-group had 9.40% distances to the hornbill used as the out group. COI sequence gene from these seven hornbill species are novel for identifying Indonesian hornbills. We encourage its use as quick species identification, applied to prevent illegal poaching conservation management.

1. Introduction

Hornbills (Aves: Bucerotidae) are group of large birds, with dominant body color of black and white; some species have casque on their beak, and dieting mostly on fruits and insects (Poonswad *et al.* 1998; MacKinnon *et al.* 2010; Poonswad *et al.* 2013; Eaton *et al.* 2016). They play an important role as seed dispersers in the forest (Kinnaird 1998; Kitamura *et al.* 2008; Balasubramanian *et al.* 2011). They are able to disperse of exotic plant seed (Viseshakul *et al.* 2011). There are 13 species of hornbills in Indonesia and all is protected by law (Sukmantoro *et al.* 2007).

As the clear identity for hornbill genus and some of its species are not yet available, resulting in the variety of names used to address the hornbill genus and its species (MacKinnon *et al.* 1998; Coates *et al.* 2000; Sukmantoro *et al.* 2007; MacKinnon *et al.* 2010; Poonswad *et al.* 2013; Eaton *et al.* 2016); while taxonomically, some species are still reluctantly categorized as sub species, such as Rhinoceros Hornbill *Buceros rhinoceros*, Oriental Pied Hornbill *Anthracoceros albirostris*, Knobbed Hornbill *Aceros cassidix*, Sulawesi Hornbill *Penelopides exarhatus*, and Blyth's Hornbill *Rhyticeros plicatus* (Coates *et al.* 2000; MacKinnon *et al.* 2010; Poonswad *et al.* 2013; Eaton *et al.* 2016). The genus and species determination are still based on mere morphological characters such as body color and body parameters. While species identification using morphological characters can be accurate for some species with sufficient records, however, it brings confusion when identification is done for species with no specific morphological characters. The molecular technic offers solution for this problem, by using reliable molecular marker for animal barcoding, which is the cytochrome oxidase sub-unit I (COI) from mitochondrial DNA (Hebert *et al.* 2003b, 2004; Hajibabaei *et al.* 2006).

The cytochrome oxidase sub-unit I gene of mitochondrial DNA (hereinafter COI gene) is a steady gene used for DNA barcoding. Mitochondrial DNA,

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with no recombination and high mutation rate during its maternal inheritance and much higher than that of the nuclear DNA, fulfills most requirements as molecular marker at the inter species level of vertebrate (Brown *et al.* 1982; Avise 1994). It is also a valuable and powerful tool for identifying molecular samples from threatened or endangered species (Palumbi and Cipriano 1998). The COI gene has been used to determine the identity of certain species (Solihin 1994) and has become satisfied genetic marker for vertebrate animal barcoding DNA with its 648 bp of nucleotide sequence length (Hebert *et al.* 2004). DNA barcoding has great contribution in taxonomic, population genetics, diversity; as well become tool in the research on level of variation among species and phylogenetic study (Hajibabaei *et al.* 2007; Imtiaz *et al.* 2017). Currently, there is no COI gene for Indonesian hornbills available and this study provides the first batch of DNA barcodes for some Indonesian hornbill species.

This study aimed to determine the genetic variation within and among Indonesian hornbill species to clarify their relationship; as well as to seek the potential use for future barcoding. The sequence of nucleotides generated from this study can be used to identify hornbills species genetically, using even small amount of DNA source samples, such as molted feathers, beaks, and other body parts.

2. Materials and Methods

2.1. Sample Collection

The research had been conducted from July 2016 to December 2017. Blood sample were collected from 31 individuals of seven hornbill species, which separatedly reared in Taman Mini Indonesia Indah (TMII) Jakarta, Taman Safari Indonesia (TSI) Cisarua Bogor, and Taman Margasatwa Ragunan (TMR) Jakarta (Table 1). As much as 0.3-1.0 ml of blood were micro-piped through the carpal joints vein, guided by the ethical approval of Bogor Agricultural University Animal Ethic Commission number 39-2106 IPB 2016. Bloods preserved following Seutin *et al.* (1991). Molecular analysis was then carried out in the Laboratory of Molecular Biology, Research Center for Biological Resources and Biotechnology

Table 1. Number of samples used for analysis

Species	English name	*n*	Sample code	Ring/microchip code	Location
Anthracoceros malayanus	Black Hornbill	4	AM1RG	985121018348577	TMR
A. malayanus	Black Hornbill		AM2RG	985121018311231	TMR
A. malayanus	Black Hornbill		AM1TM	TMII12048	TMII
A. malayanus	Black Hornbill		AM1TS	TSIBGR12G 038	TSI
Anthracoceros albirostris	Oriental Pied Hornbill	6	AA1RG	985121018247394	TMR
A. albirostris	Oriental Pied Hornbill		AA1TM	TMIIK 12013	TMII
A. albirostris	Oriental Pied Hornbill		AA2TM	K12046	TMII
A. albirostris	Oriental Pied Hornbill		AA3TM	K12047	TMII
A. albirostris	Oriental Pied Hornbill		AA5TS	TSIBGR12G 016	TSI
A. albirostris	Oriental Pied Hornbill		AA11TS	TSIBGR12G 018	TSI
Aceros cassidix	Knobbed Hornbill	3	AC1RG	985121018260062	TMR
A. cassidix	Knobbed Hornbill		AC1TS	TSIBGR17G 047	TSI
A. cassidix	Knobbed Hornbill		AC2TS	TSIBGR17G 046	TSI
Rhyticeros plicatus	Blyth's Hornbill	5	RP1RG	985121018304960	TMR
R. plicatus	Blyth's Hornbill		RP1TM	N365	TMII
R. plicatus	Blyth's Hornbill		RP2TM	213	TMII
R. plicatus	Blyth's Hornbill		RP1TS	TSIBGR14G 048	TSI
R. plicatus	Blyth's Hornbill		RP3TS	TSIBGR17G 065	TSI
Rhyticeros undulatus	Wreathed Hornbill	8	RU1TM	I113	TMII
R. undulatus	Wreathed Hornbill		RU2TM	N312	TMII
R. undulatus	Wreathed Hornbill		RU3TM	N325	TMII
R. undulatus	Wreathed Hornbill		RU4TM	242	TMII
R. undulatus	Wreathed Hornbill		RU5TM	N346	TMII
R. undulatus	Wreathed Hornbill		RU6TM	N298	TMII
R. undulatus	Wreathed Hornbill		RU7TM	ZTMII115	TMII
R. undulatus	Wreathed Hornbill		RU5TS	TSIBGR17G 061	TSI
Buceros rhinoceros	Rhinoceros Hornbill	4	BR1TM	N324	TMII
B. rhinoceros	Rhinoceros Hornbill		BR2TM	N300	TMII
B .rhinoceros	Rhinoceros Hornbill		BR1RG	985121018297670	TMR
B. rhinoceros	Rhinoceros Hornbill		BR2TS	TSIBGR17G 044	TSI
Buceros bicornis	Great Hornbill	1	BB1RG	985121018259627	TMR
Aceros waldeni	Rufous headed Hornbill	1	NC015085	-	GenBank
A. corrugatus	Wrinkled Hornbill	1	HM755883	-	GenBank
Penelopides panini	Visayan Tarictic Hornbill	1	NC015087	-	GenBank
Total of sample		34			

n= number of individuals, tmr= taman margasatwa ragunan, tsi= taman safari indonesia, tmii= taman mini ndonesia Indah

(PPSHB), Bogor Agricultural University (IPB). Three COI gene sequences downloaded from GenBank were used as the out group (NC015085, HM755883, and NC015087).

2.2. Isolation and Purification

Blood sample from 31 individuals were preserved in 100% ethanol and freezing at -20°C. As much as 25 mg of blood was put into the effendorf tube and washed by Tris-EDTA buffer (*low TE*). Genome DNA was isolated with Spin-Column Protocol, using DNeasy Tissue Kit ® Blood and paint No. 69 504 (50) from Qiagen.

2.3. PCR Amplification and Sequencing

We used polymerase chain reaction (PCR) technique to identify differences in the COI gene nucleotide sequences. Primer was designed using Primer3 software (http://bio-info.ut.ee/primer3-0.4.0/primer3), where the alignment was based on the one of Rufous-headed Hornbill *Aceros waldeni* (accession no. NC015085). The primers were named as COIBuceF (5'-TCAACTAACCACAAAGACATCGGCAC-3') and COIBuceR (5'-ACGTGTGAGATAATTCCAAAGCCTG-3') and produced 746 bp nucleotides. Amplification was performed in Rotor Gene-Q machine.

Reaction mixture consisted of 2 µl DNA template, 1.0 µl forward and reverse primer (20 pmol/µl), 6.8µl ddH2O, 5.0 µl Qs buffer, 5.0 µl Enhancer, 1.0 µl dNTP, and 0.2 µl Taq polymerase. The PCR performed temperature sequences as follow; 95°C of pre-denaturation (5 minutes), 94°C of denaturation (1 minute), annealing with 54°C (45 seconds), and extension at 72°C temperature (1 minutes). Amplified DNA were then migrated onto 1.2% agars gel (Sambrook 1989), before transported to First BASE laboratory in Malaysia for further sequencing.

2.4. Data Analysis

Nucleotides sequences were edited and aligned using Clustal W implemented in MEGA 6.0 software (Tamura *et al.* 2013). We used BIOEDIT version 7.0.9 (Hall 1999) for checking and trimming the sequences. The final alignment of each samples (746 bp) was then imported into Barcode of Life Database (BoLD) System in http://www.barcodinglife.org website to determine the similarity of the samples. Genetic distances were determined based on Kimura 2-parameter (K2P) method (Kimura 1980). We reconstructed phylogenetic tree using Neighbor-Joining (NJ) models with 1000 bootstrap repetition (Tamura *et al.* 2013).

3. Results

3.1. Nucleotide Variation

Variation, mutation type and basic composition from 31 individuals of the seven Indonesian hornbills species were summarized in Table 2. Final alignment amplified COI gene with Clustal W of MEGA 6 software resulting in total of 746 bp. The number of conservative sites on genus level ranged between 712 bp (95.44%) and 737 bp (98.79%), and on family was 638 bp (85.53%). The variable sites for genera *Aceros, Rhyticeros, Buceros,* and *Anthracoceros* were as many as 9, 16, 17, and 34 sites respectively. The variable sites for the whole sample (Bucerotidae family) were 108 bp (14.47%).

The most number of singleton sites in genus level was observed on *Buceros* genus (17 bp) and the least was at *Rhyticeros* (5 bp). The most informative Parsimony site was in *Anthracoceros* genus (24 bp) and the lowest one on *Aceros* (0 bp). Number of transitional substitutions (21 bp) were greater than transversion substitutions (9 bp).

Table 2. Conserve and variable sites, types of mutations, and base composition of the Indonesian hornbills COI gene in 746 bp length

Taxa	*n*	Conserve sites	Variables		Total of variable sites	si			sv			R	Base composition			
			Pi	s		1st	2nd	3rd	1st	2nd	3rd		A	T	G	C
Genus																
Aceros	3	737	0	9	9	1	1	1	1	0	2	0.8	26.8	27.2	16.4	29.6
Rhyticeros	13	730	11	5	16	2	0	3	1	0	1	3.7	26.7	26.1	16.5	30.8
Buceros	5	727	2	17	19	1	0	2	2	0	2	0.6	27.0	25.7	16.3	31.0
Anthracoceros	10	712	24	10	34	3	0	6	1	1	2	2.3	26.7	25.7	15.6	31.9
Family																
Bucerotidae	31	638	79	29	108	4	1	16	2	1	6	2.5	26.8	26.0	16.2	31.0

n= sample number, pi= parsimony-informative site, s= singleton site, si= transitional pairs, sv= transversion pairs, r= ratio of si/sv

Transition and transversion substitution occured at the third codon for both genus and family level. The ratio of transitional and transversional pairs values (R) ranged between 0.8 and 3.7 on genus level, and 2.5 on family level. Cytosine (C) became the most nucleotide in COI gene sequences composition and guanine (G) was the lowest one. AT base composition for all samples were 52.8% and GC 47.2%.

3.2. Species Identification

We presented similarity scores of 31 hornbill individuals examined with BoLD System in Table 3 where each individual paired with the three hornbills in out-group and ranked according to the degree of its similarity. We observed the three highest similarity score among paired individuals; the first ranged between 91.05% and 92.89%, the

Table 3. Top three species identification result based on BoLD system database

Species	Sample code	Top three species	Similarity (%)	Species	Sample code	Top three species	Similarity (%)
Anthracoceros malayanus	AM1RG	*P. panini*	91.51	*B. rhinoceros*	BR2TS	*P. panini*	91.67
		A. coronatus	90.69			*A. waldeni*	89.48
		A. waldeni	89.88			*A. coronatus*	89.34
A. malayanus	AM2RG	*P. panini*	91.51	*Buceros bicornis*	BB1RG	*P. panini*	91.67
		A. coronatus	90.69			*A. waldeni*	90.03
		A. waldeni	90.15			*R. leucocephalus*	89.78
A. malayanus	AM1TM	*P. panini*	91.67	*Rhyticeros undulatus*	RU1TM	*P. panini*	92.58
		A. coronatus	90.69			*A. waldeni*	91.63
		A. waldeni	90.15			*R. corrugatus*	91.55
A. malayanus	AM1TS	*P. panini*	91.05	*R. undulatus*	RU2TM	*P. panini*	92.58
		A. coronatus	90.28			*A. waldeni*	91.63
		A. waldeni	89.47			*R. corrugatus*	91.55
Anthracoceros albirostris	AA1RG	*A. coronatus*	92.04	*R. undulatus*	RU3TM	*P. panini*	92.71
		P. panini	91.36			*A. waldeni*	91.77
		R. corrugatus	89.83			*R. corrugatus*	91.71
A. albirostris	AA1TM	*A. coronatus*	92.04	*R. undulatus*	RU4TM	*P. panini*	92.75
		P. panini	91.36			*A. waldeni*	91.90
		R. corrugatus	89.83			*R. corrugatus*	91.55
A. albirostris	AA2TM	*A. coronatus*	92.04	*R. undulatus*	RU5TM	*P. panini*	92.75
		P. panini	91.36			*A. waldeni*	91.90
		R. corrugatus	89.83			*R. corrugatus*	91.55
A. albirostris	AA3TM	*A. coronatus*	91.90	*R. undulatus*	RU6TM	*P. panini*	92.71
		P. panini	91.23			*A. waldeni*	91.77
		R. corrugatus	89.83			*R. corrugatus*	91.71
A. albirostris	AA5TS	*A. coronatus*	91.77	*R. undulatus*	RU7TM	*P. panini*	92.75
		P. panini	91.09			*A. waldeni*	91.90
		A. waldeni	89.74			*R. corrugatus*	91.77
A. albirostris	AA11TS	*A. coronatus*	91.90	*R. undulatus*	RU5TS	*P. panini*	92.59
		P. panini	91.23			*A. waldeni*	91.77
		R. corrugatus	89.67			*R. corrugatus*	91.39
Aceros cassidix	AC1RG	*P. panini*	92.56	*Rhyticeros plicatus*	RP1RG	*P. panini*	92.98
		R. corrugatus	91.67			*A. waldeni*	91.50
		A. waldeni	91.46			*R. corrugatus*	91.39
A. cassidix	AC1TS	*P. panini*	92.75	*R. plicatus*	RP1TM	*P. panini*	92.71
		R. corrugatus	91.55			*A. waldeni*	91.23
		A. waldeni	91.36			*R. corrugatus*	91.08
A. cassidix	AC2TS	*P. panini*	92.44	*R. plicatus*	RP2TM	*P. panini*	92.98
		R. corrugatus	91.24			*A. waldeni*	91.50
		A. waldeni	91.09			*R. corrugatus*	91.39
Buceros rhinoceros	BR1TM	*P. panini*	91.94	*R. plicatus*	RP1TS	*P. panini*	92.85
		A. waldeni	89.75			*A. waldeni*	91.36
		A. coronatus	89.62			*R. corrugatus*	91.24
B. rhinoceros	BR2TM	*P. panini*	92.08	*R. plicatus*	RP3TS	*P. panini*	92.85
		A. waldeni	89.89			*R. corrugatus*	91.55
		A. coronatus	89.75			*A. waldeni*	91.36
B. rhinoceros	BR1RG	*P. panini*	92.08				
		A. waldeni	89.89				
		A. coronatus	89.75				

R. corrugatus= Rhabdotorrhinus corrugatus, R. leucocephalus= Rhabdotorrhinus leucocephalus, A. coronatus= Anthracoceros coronatus

second ranged between 89.48% and 91.90%, and the third between 89.34% and 91.77%. Five hornbill species fell within those ranges, they are *Penelopides panini, Aceros waldeni, A. coronatus, Rhabdotorrhinus (Aceros) corrugatus* and *R. leucocephalus.*

3.3. Single Nucleotide Polymorphism within Genus and Family

This study detected variations of nucleotides within genus and family. Alignment result of COI gene sequence from 31 hornbill samples indicated 45 sites of single nucleotide polymorphism (SNP) within genus dan family species (Table 4) which located between 84 and 737 sites. Number of different sites between species (within genus) was ranged from 2-7 sites. The *Anthracoceros malayanus* has 7 distinguishing specific nucleotide sites with other species, positioned at site 108, 378, 537, 604, 642, 717, and 718. The *A. albirostris* has 5 different nucleotide specific sites with other species, located at site 373, 471, 597, 640, and 697. The *Buceros rhinoceros* has 2 specific nucleotide sites with other species, located at site 339 and 510.

The *B. bicornis* has 3 different specific nucleotide sites with other species, positioned at site 240, 241, and 579. The *Aceros cassidix* has 6 distinct specific nucleotide sites with other species, founded at site 84, 90, 219, 303, 603, and 690. The *Rhyticeros undulatus* and *R. plicatus* have only 2 and 3 different sites each (Table 4). We also found the different specific nucleotide site within family or between genera. Number of different specific nucleotide sites between genera was ranged from 0-10 sites. The *Anthracoceros* genus has 10 divergent sites with other genera, positioned at 222, 255, 261, 345, 453, 519, 594, 600, 615, and 712 sites. The *Buceros* genus has 5 divergent sites with other genera, and the *Aceros* genus has 6 different sites. However, *Rhyticeros* has no different site at genus level (Table 4).

3.4. Genetic Distance

Genetic distance within species, genus, and family of hornbills was measured using K2P methods (Table 5 and 6). Average genetic distance within species was ranged from 0.002 (0.2%) to 0.008 (0.8%), within genus 0.045 (4.5%), and within family 0.046 (4.6%) (Table 5).

The mean genetic distance within genus appears to vary (Table 6). Genetic distance between *Rhyticeros undulatus* and *R. plicatus* was 0.013 (1.3%) and between *Buceros rhinoceros* and *B. bicornis* 0.016 (1.6%). Interspecific genetic distance within two genera (*Rhyticeros* and *Buceros*) was lower than the threshold for species separation (> 3.0%). Genetic distance within Bucerotidae family 0.046 (4.6%), which is lower than what found in previous studies (Table 6).

3.5. Phylogenetic

We reconstructed phylogenetic tree using NJ models with 1000 bootstrap repetition (Figure 1). Three hornbill species were retrieved from GenBank (NCBI) as an out group. In the tree, Indonesian hornbills lumped into two main groups (clades) with bootstrap ranged from 54 to 100%. Group I consisted of five species, namely *Aceros cassidix*, *Rhyticeros plicatus, R. undulatus, Buceros rhinoceros,* and *B. bicornis* with bootstrap value ranged from 54 to 99%. Two members of *Anthracoceros, A. malayanus* and *A. albirostris*, formed separate Group II, with bootstrap 88-100%. The two groups were supported by 0.059 (5.90%) genetic distance. Indonesian hornbills clearly separated from hornbills in the out group, supported with 0.094 (9.40%) genetic distance.

4. Discussion

Small nucleotide variation was found within species level, while fairly large difference was shown within genus and family level (Tabel 2). The length of the COI gene used for the barcode is around half of the total length located at beginning of the COI gene in all animal species (Pacheco *et al.* 2011; Sammler *et al.* 2011; Gonzales *et al.* 2013; Zhou *et al.* 2015). Very short barcode sequences (109-208 bp) are, nonetheless, also useful for species identification (Hajibabaei *et al.* 2006). In our research, the COI gene was successfully amplified into the length of 746 bp. It is longer than the one suggested for DNA barcode purpose (Hebert *et al.* 2004; Hajibabaei *et al.* 2006; Prehadi *et al.* 2015; Huang and Tu 2016). Nevertheless, it is shorter than Cockatoos (Psittaciformes) COI gene (807 bp) (Astuti and Sulandari 2010).

Table 4. Single nucleotide polymorphism within hornbill genus and family

Species	Sample code	Nucleotide base site																																										
		84	90	108	201	219	222	228	240	241	253	255	261	303	339	344	345	365	373	378	382	384	417	424	453	471	495	510	519	537	579	594	597	600	603	604	615	621	640	642	690	697	712	717	718	737
*Aceros waldeni**	NC015085	C	T	A	T	A	T	C	C	G	G	C	A	T	C	G	T	C	G	C	T	A	T	C	G	C	C	A	T	C	A	C	C	G	C	C	G	A	C	G	C	C	T	C	C	T
Anthracoceros malayanus	AM1RG	.	C	C	T	.	C	.	.	.	.	C	C	C	.	.	C	.	.	T	.	.	.	T	T	.	.	.	C	A	.	T	.	G	.	T	A	.	.	G	.	.	C	T	T	.
A. malayanus	AM2RG	.	C	C	T	.	C	.	.	.	.	C	C	C	.	.	C	.	.	T	.	.	.	T	T	.	.	.	C	A	.	T	.	G	.	T	A	.	.	G	.	.	C	T	T	.
A. malayanus	AM1TM	.	C	C	T	.	C	.	.	.	.	C	C	C	.	.	C	.	.	T	.	.	.	T	T	.	.	.	C	A	.	T	.	G	.	T	A	.	.	G	.	.	C	T	T	.
A. malayanus	AM1TS	.	C	C	T	.	C	.	.	.	.	C	C	C	.	.	C	.	.	T	.	.	.	T	T	.	.	.	C	A	.	T	.	G	.	T	A	.	.	G	.	.	C	T	T	.
Anthracoceros albirostris	AA1RG	.	C	.	C	.	C	.	.	.	.	C	C	C	.	.	C	.	A	.	.	.	.	T	T	T	.	.	C	.	.	T	T	G	.	.	A	.	T	A	.	T	C	.	.	.
A. albirostris	AA1TM	.	C	.	C	.	C	.	.	.	.	C	C	C	.	.	C	.	A	.	.	.	.	T	T	T	.	.	C	.	.	T	T	G	.	.	A	.	T	A	.	T	C	.	.	.
A. albirostris	AA2TM	.	C	.	C	.	C	.	.	.	.	C	C	C	.	.	C	.	A	.	.	.	.	T	T	T	.	.	C	.	.	T	T	G	.	.	A	.	T	A	.	T	C	.	.	.
A. albirostris	AA3TM	.	C	.	C	.	C	.	.	.	.	C	C	C	.	.	C	.	A	.	.	.	.	T	T	T	.	.	C	.	.	T	T	G	.	.	A	.	T	A	.	T	C	.	.	.
A. albirostris	AA5TS	.	C	.	C	.	C	.	.	.	.	C	C	C	.	.	C	.	A	.	.	.	.	T	T	T	.	.	C	.	.	T	T	G	.	.	A	.	T	A	.	T	C	.	.	.
A. albirostris	AA11TS	.	C	.	C	.	C	.	.	.	.	C	C	C	.	.	C	.	A	.	.	.	.	T	T	T	.	.	C	.	.	T	T	G	.	.	A	.	T	A	.	T	C	.	.	.
Buceros rhinoceros	BR1TM	.	C	.	C	.	.	.	.	.	A	T	.	C	T	A	.	A	.	.	.	G	C	T	A	.	.	G	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	C
B. rhinoceros	BR2TM	.	C	.	C	.	.	.	.	.	A	T	.	C	T	A	.	A	.	.	.	G	C	T	A	.	.	G	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	C
B. rhinoceros	BR1RG	.	C	.	C	.	.	.	.	.	A	T	.	C	T	A	.	A	.	.	.	G	C	T	A	.	.	G	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	C
B. rhinoceros	BR2TS	.	C	.	C	.	.	.	.	.	A	T	.	C	T	A	.	A	.	.	.	G	C	T	A	.	.	G	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	C
Buceros bicornis	BB1RG	.	C	.	C	.	.	.	G	C	A	T	.	C	.	A	.	A	.	.	.	G	C	T	A	.	.	.	.	.	C	.	.	A	.	.	.	.	.	A	.	.	.	.	.	C
Aceros cassidix	AC1RG	T	T	.	C	G	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	.	.	.	A	A	.	.	.	.	A	T	.	.	.	.	.
A. cassidix	AC1TS	T	T	.	C	G	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	.	.	.	A	A	.	.	.	.	A	T	.	.	.	.	.
A. cassidix	AC2TS	T	T	.	C	G	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	.	.	.	A	A	.	.	.	.	A	T	.	.	.	.	.
Rhyticeros undulatus	RU1TM	.	C	.	C	.	.	A	.	.	.	T	.	C	.	.	.	.	.	.	C	.	.	T	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.
R. undulatus	RU2TM	.	C	.	C	.	.	A	.	.	.	T	.	C	.	.	.	.	.	.	C	.	.	T	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.
R. undulatus	RU3TM	.	C	.	C	.	.	A	.	.	.	T	.	C	.	.	.	.	.	.	C	.	.	T	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.
R. undulatus	RU4TM	.	C	.	C	.	.	A	.	.	.	T	.	C	.	.	.	.	.	.	C	.	.	T	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.
R. undulatus	RU5TM	.	C	.	C	.	.	A	.	.	.	T	.	C	.	.	.	.	.	.	C	.	.	T	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.
R. undulatus	RU6TM	.	C	.	C	.	.	A	.	.	.	T	.	C	.	.	.	.	.	.	C	.	.	T	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.
R. undulatus	RU7TM	.	C	.	C	.	.	A	.	.	.	T	.	C	.	.	.	.	.	.	C	.	.	T	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.
R. undulatus	RU5TS	.	C	.	C	.	.	A	.	.	.	T	.	C	.	.	.	.	.	.	C	.	.	T	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.
Rhyticeros plicatus	RP1RG	.	C	.	C	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	C	A	.	G	.	.	.	.	.	.	A	.	.	.	G	.	A	.	.	.	.	.	.
R. plicatus	RP1TM	.	C	.	C	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	C	A	.	G	.	.	.	.	.	.	A	.	.	.	G	.	A	.	.	.	.	.	.
R. plicatus	RP2TM	.	C	.	C	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	C	A	.	G	.	.	.	.	.	.	A	.	.	.	G	.	A	.	.	.	.	.	.
R. plicatus	RP1TS	.	C	.	C	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	C	A	.	G	.	.	.	.	.	.	A	.	.	.	G	.	A	.	.	.	.	.	.
R. plicatus	RP3TS	.	C	.	C	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	C	A	.	G	.	.	.	.	.	.	A	.	.	.	G	.	A	.	.	.	.	.	.

*= GenBank (NCBI) database. Numbers below nucleotide base site names are site numbers for each base change in our alignment for COI gene. Site one in our alignment is equivalent to site 28 in the full Aceros waldeni sequence (accession no. NC01508)

Table 5. Average genetic distance within species, genus, and family of hornbills based on partial COI gene in 746 length

Genetic distance	*A. malayanus*	*A. albirostris*	*B. bicornis*	*B. rhinoceros*	*R. undulatus*	*R. plicatus*	*A. cassidix*
Within species minimum	0.001	0.000	-	0.001		0.000	0.003
Within species maximum	0.011	0.007	-	0.011		0.004	0.012
Mean within species	0.007	0.004	-	0.007		0.002	0.008
Within genus minimum					0.013		
Within genus maximum					0.066		
Mean within genus					0.045		
Within family minimum					0.028		
Within family maximum					0.062		
Mean within family					0.046		

Table 6. Genetic distance within genus of bucerotidae

Species	1	2	3	4	5	6	7	8	9	10
A. malayanus										
A. albirostris	0.032									
B. bicornis	0.063	0.054								
B. rhinoceros	0.061	0.054	0.016							
R. undulatus	0.065	0.055	0.036	0.041						
R. plicatus	0.063	0.052	0.033	0.038	0.013					
A. cassidix	0.066	0.060	0.044	0.052	0.030	0.027				
A. waldeni	0.110	0.112	0.108	0.115	0.088	0.093	0.094			
A. corrugatus	0.104	0.103	0.106	0.109	0.086	0.085	0.049	0.049		
10. P. panini	0.100	0.093	0.090	0.087	0.078	0.075	0.085	0.059	0.052	

We presented similarity scores of 31 hornbill individuals examined with BoLD System in Table 3 where each individual paired with the three hornbills in out-group and ranked according to the degree of its similarity. Five hornbill species fell within those ranges, they are *Penelopides panini, Aceros waldeni, A. coronatus, Rhabdotorrhinus (Aceros) corrugatus,* and *R. leucocephalus*. These species belong to the family of Bucerotidae order Bucerotiformes (Poonswad *et al.* 2013).

All tested individuals was having similarity values less than 97.0%. This fact reflected the inavailability of nucleotide sequence of the COI genes of these seven hornbills species in BoLD System database. These hornbills had 7.11% difference to hornbill database in BoLD System. The difference here is considerably higher than the interspecific threshold among animal species (> 3.0%) (Hebert *et al.* 2003a; Vilaça *et al.* 2006; Efe *et al.* 2009). Therefore, the COI gene nucleotide sequences studied is a new data and could be used as a reference in identifying of Indonesian hornbill.

This study identified variations of nucleotides within genus and family. Alignment result of COI gene sequence from 31 hornbill samples indicated 45 sites of single nucleotide polymorphism (SNP) within genus dan family species (Table 4) which located between 84 and 737 sites. At species level, we detected three hornbills (*Anthracoceros malayanus, A. albirostris*, and *Aceros cassidix*) with the highest variation, while the rest of the species lack with variation. At the genus level, the highest variation of COI gene was found in *Anthracoceros* (10 sites) and the lowest was at *Buceros* (5 sites). Variations in COI gene sequences can be used to distinguish close-allied species in all animal taxa which suggested that the diversity of mtDNA COI gene sequences in 648 bp areas may potentially function as animal species barcodes (Hebert *et al.* 2004), and each species has a specific nucleotide sequence in the COI gene (Waugh 2007). Genetic variation at the DNA level can be identified through modern molecular technology (Sutarno 2003).

Genetic distance within species, genus, and family of hornbills was measured using K2P methods (Table 5 and 6). Average genetic distance within species was ranged from 0.002 (0.2%) to 0.008 (0.8%), within genus 0.045 (4.5%), and within family 0.046 (4.6%) (Table 5). Genetic distance distinguishing within species were lower than those learnt from previous studies (Yoo *et al.* 2006; Astuti and Sulandari 2010; Huang and Tu 2016); yet, it remains the same on genus and

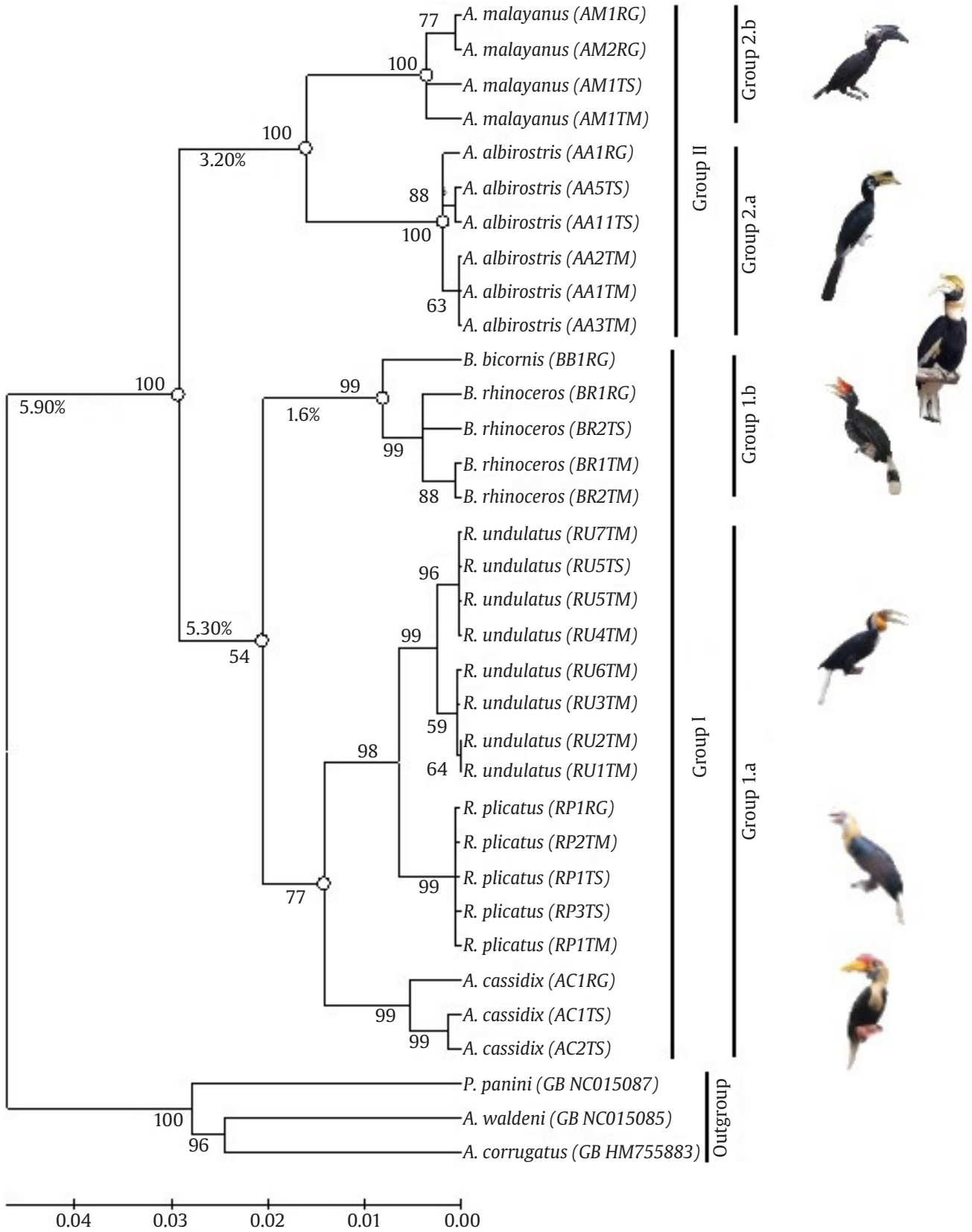

Figure 1. Neighbor-joining (NJ) phylogenetic tree of hornbills based on partial sequence of COI gene with 746 bp length

family level, especially to what observed in Hebert *et al.* (2004), Kerr *et al.* (2007), Astuti and Sulandari (2010), and Huang and Tu (2016). Genetic distance within species is usually less than 1% and infrequently reaching more than 2% (Waugh 2007), as seen in average genetic distance within species of Korean birds and Phasianidae as much as 0.3% (Yoo *et al.* 2006; Cai *et al.* 2010), in Neotropical birds which ranged between 0 to 13.7% (Tavares *et al.* 2011) and in parrots that ranged from 0.1 to 0.7% (Gonçalves *et al.* 2015).

The mean genetic distance within genus appears to vary (Table 6). Interspecific genetic distance within two genera (*Rhyticeros* and *Buceros*) was lower than the threshold for species separation (> 3.0%). According to Efe *et al.* (2009) the difference in COI gene among species within Laridae (Sternini) ranged from 0.25 to 10.51%. In addition, *Rhyticeros undulatus* and *R. plicatus* have some morphological similarities. Variations in their body color are found in the head, neck, and neck pouch. The pair of *Buceros rhinoceros* and *B. bicornis* also bear some resemblances, where both having identical shape of casque, similar color on neck and wings (MacKinnon *et al.* 2010; Poonswad *et al.* 2013; Eaton *et al.* 2016). In contrast, genetic distance between *Anthracoceros malayanus* and *A. albirostris* was 0.032 (3.2%), as the interspecific COI sequence differences on *Anthracoceros* genus is above the threshold. The genetic distance to accurately identify species should be above 5% (Waugh 2007). Huang and Tu (2016) reported the mean divergence of the COI gene within genus in Ardeidae was 13.08%; 8.2% within genera of Korean birds (Yoo *et al.* 2006); 4.8-15.6% within Thamnophilidae (Passeriformes) (Vilaça *et al.* 2006); 5.35% on Phasianidae (Cai *et al.* 2010); 7.95% on Scandinavian birds (Johnsen *et al.* 2010); and 9.52% on Green Bee-eater (Merops orientalis) (Arif *et al.* 2011). Genetic distance within Bucerotidae family 0.046 (4.6%), which is lower than what found in previous studies (Table 6). Yoo *et al.* (2006) found mean genetic distance within the Korean bird families was 13.8%, while on Phutananidae was 15.63% (Cai *et al.* 2010).

Our phylogenetic tree delineated the segregation of genus Anthracoceros from other genera tested in this study. The seven species of Indonesian hornbill clustered accordingly in phylogeny tree. This study indicated that phylogenetic tree reconstructed from COI genes is deemed to be able to separate interspecies (within genus) and inter genera (within family) of hornbills. It also suggested that the kinship test based on COI gene sequence has considerable accuracy. Branches in phylogenetic trees represent the relationships between units, describing hereditary relationships back to the ancestors, while the length of branches describes the number of evolutionary changes occurred between two nodes (Graur and Li 2000). Sequence diversity of COI genes (648 bp) retains potential use for identification on species level, as well as having function as DNA barcode (Hebert *et al.* 2003a, b; Hebert and Gregory 2005). DNA barcoding based on COI has been successful in species determination and phylogeny across animal species (Huang and Tu 2016), as well proven satisfied on animal identification (Hajibabaei *et al.* 2006).

Acknowledgements

We would like to thank to the Directorate General of Higher Education, Ministry of Research, Technology, and Higher Education of the Republic Indonesia for funding this research through doctoral dissertation and applied research of higher education research grant on 2017 (contract number 896/UN30.15/LT/2017 and 011/SP2H/LT/DRPM/VIII/2017). This research has been approved by Director General of Natural Resources Conservation and Ecosystem, Ministry of Environmental and Forestry, Number: SK.399/KSDAE/SET/KSA.2/11/2016. We also thank to the animal ethics commission of the Bogor Agricultural University for ethic approval. We grateful to LIPI and BKSDA Jakarta for the SATS-DN recommendation and sampling permit in TMII and TMR. We also thank to M. Nazri Janra who helped us to improve the English of this manuscript. The authors are grateful to TMII, TMR, TSI staff, and our laboratory collogues for their assistance in this study.

Conflict of Interest

There is no conflict of interest among authors.

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