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CONTENT

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Pipit Pitriana

Exploring sponge-inhabiting barnacles of eastern Indonesia using micro-CT scanning

TREUBIA, December 2020, Vol. 47, No. 2, pp. 77–98.

We present a morphological study of Indonesian sponge-inhabiting barnacles using standard light microscopy in combination with micro-CT scanning and computer-aided 3D-reconstruction of the external shell morphology. A taxonomic analysis of the material detected four different genera of sponges inhabited by five different species of balanomorph barnacles, two of which are undescribed. Together with conventional morphological examination by dissection, we provide modern non-destructive imaging methods, using micro-CT scanning to enhance our knowledge of the morphological characters of sponge-inhabiting barnacles from eastern Indonesia. Although there were some methodological limitations regarding the contrast-enhancing technique, this study demonstrates micro-CT as a useful non-destructive technique of integrative taxonomy, for the examination of sponge-inhabiting barnacles.

(Pipit Pitriana, Andreas Wessel, Tina Aschenbach, and Kristina von Rintelen)

Keywords: Cirripedia, Indonesian biodiversity, integrative taxonomy, micro-computer tomography, shell morphology

UDC: 598.89:577.2

Jarulis

Characters of mitochondrial DNA D-loop hypervariable III fragments of Indonesian Rhinoceros Hornbill (*Buceros rhinoceros*) (Aves: Bucerotidae)

TREUBIA, December 2020, Vol. 47, No. 2, pp. 99–110.

The Rhinoceros Hornbill (*Buceros rhinoceros*) genetic characteristics consist of nucleotide polymorphisms, haplotypes, genetic distances, and relationships which are important for their conservation effort in Indonesia. We sequenced mitochondrial DNA D-loop hypervariable III fragments from five Rhinoceros Hornbill individuals at Safari Park Indonesia I and Ragunan Zoo, which were isolated using Dneasy® Blood and Tissue Kit Spin-Column Protocol, Qiagen. D-loop fragment replication was done by PCR technique using DLBuce_F (5'-TGGCCTTTCTCCAAGGTCTA-3') and DLBuce_R (5'-TGAAGG AGT TCATGGGCTTAG-3') primer. Thirty SNP sites were found in 788 bp D-loop sequences of five Rhinoceros Hornbill individuals and each individual had a different haplotype. The average genetic distance between individuals was 3.09% and all individuals were categorized into two groups (Group I: EC6TS, EC1RG, EC2TS and Group II: EC9TS, EC10TS) with a genetic distance of 3.99%. This result indicated that the two groups were distinct subspecies. The genetic distance between Indonesian and Thai Rhinoceros Hornbills was 10.76%. Five Indonesian Rhinoceros Hornbill individuals at Safari Park Indonesia I and Ragunan Zoo probably came from different populations, ancestors, and two different islands. This study can be of use for management consideration in captive breeding effort at both zoos. The D-loop sequence obtained is a

useful character to distinguish three Rhinoceros Hornbill subspecies in Indonesia.

(Jarulis, Choirul Muslim, Dedy Duryadi Solihin, Ani Mardiastuti, and Lilik Budi Prasetyo)

Keywords: Bucerotidae, control region, phylogenetic, Rhinoceros Hornbill conservation, zoo

UDC: 595.773.4(594.59)

Eka Kartika Arum Puspita Sari

Diversity of fruit flies (Tephritidae: *Bactrocera* spp.) in campus C of Airlangga University, Surabaya, Indonesia

TREUBIA, December 2020, Vol. 47, No. 2, pp. 111–122.

This research aims to get information about the species of host plants and fruit flies, composition and structure of community, distribution pattern, and impact of environmental factors to fruit flies in Campus C, Airlangga University. Research was conducted from August to November 2019. A modification of Steiner trap with methyl eugenol 1.5 ml bait was installed in nine sites. Each Steiner trap was placed on a mango tree 1-2 meters above ground level. Trapped fruit fly specimens were collected after one week. Four replications were made, with intervals between two periods of installation. As many as 682 host plants of the fruit flies were found at the study site consisting of 25 species from 15 families. Results showed that 1121 individuals of *Bactrocera* fruit flies were found, consisting of 5 species, namely *B. carambolae*, *B. dorsalis*, *B. minuscula*, *B. occipitalis*, and *B. musae*. The most abundant species was *B. carambolae* (62.8%), followed by *B. dorsalis* (27.3%), *B. minuscula* (8.4%), *B. occipitalis* (1%), and the lowest was *B. musae* (0.5%). *B. occipitalis* has an even distribution pattern, while four other species have aggregated distribution patterns. The diversity index at nine locations ranged from 0.772 (low) to 1.151 (moderate). *B. carambolae* and *B. dorsalis* were the dominant species. The presence of fruit flies was influenced by environmental (humidity, temperature, sunlight intensity, wind) and host plant factors.

(Eka Kartika Arum Puspita Sari, Moch. Affandi, and Sucipto Hariyanto)

Keywords: Dacinae, diversity, fruit flies, methyl eugenol, Steiner trap

UDC: 595.799:598.836:591.5(594.5)

Sih Kahono

First report on hunting behavior of migratory Oriental Honey-buzzard (*Pernis ptilorhynchus orientalis*) towards migratory giant honeybee (*Apis dorsata dorsata*) (Hymenoptera: Apidae) on Java Island, Indonesia

TREUBIA, December 2020, Vol. 47, No. 2, pp. 123–132.

Both migratory Oriental Honey-buzzard (*Pernis ptilorhynchus orientalis*) and migratory giant honeybee (*Apis dorsata dorsata*) can be found in South-east Asia. The Oriental Honey-buzzard is the main predator of the giant honeybee, prey upon its honeycomb, larvae, and honey. Its existence always follows the migration of the giant honeybee. They stay on Java island during the migratory season. The giant honeybee lives in a large colony and has a powerful sting that is useful for defence against its predators. The bee is among the most dangerous animals since its threatening defensive behavior causes severe impact on the eagle and is even frequently fatal for human beings. Data collections on hunting behavior of the Oriental Honey-buzzard were based on irregular observations and interviews between the year 2003 to 2019. We categorized five hunting behaviors during data collections: flying orientation around the bee's nest, attack on living nest, failure to collect the living nest, preying upon the newly empty nest, and transferring attack of the angry bee to people nearby. The safest hunting for the Oriental Honey-buzzard is to prey upon newly empty nest left by the honeybee. When the nest was still occupied by the bee colonies, the eagle should develop a strategy to avoid and reduce the risk of being attacked. It sometimes transfers the attack to people nearby.

(Sih Kahono, Dewi M. Prawiradilaga, Djunijanti Peggie, Erniwati, and Eko Sulistyadi)

Keywords: hunting behavior, Java, migratory giant honeybee, Oriental Honey-buzzard

UDC: 595.798:57.06(594.4)

Fuki Saito-Morooka

Taxonomic notes on the hover wasp genus *Eustenogaster* (Vespidae, Stenogastrinae), with description of two new species from Sumatra Island, Indonesia

TREUBIA, December 2020, Vol. 47, No. 2, pp. 133–154.

Wasps of the genus *Eustenogaster* van der Vecht, 1969, with 17 species currently recognized, are distributed from the Indian subcontinent in the west to the Philippines, Sulawesi Island and Java Island in the east. Two new species of hover wasp genus *Eustenogaster* (*E. multifolia* **sp. nov.**, *E. sumatraensis* **sp. nov.**) are described from specimens collected in Sumatra Island. The female of *E. vietnamensis* occurring in Vietnam are described for the first time. The lectotypes of *Paravespa eva* Bell, 1936 and *Ischnogaster ornatifrons* Cameron, 1902 are designated. The new taxonomic status is proposed for *Stenogaster eximioides* Dover and Rao, 1922 as a good (=valid) species of *Eustenogaster*. The synonymy of *Ischnogaster ornatifrons* Cameron, 1902 with *Eustenogaster micans* (de Saussure, 1852) has been confirmed. A revised key to species and a taxonomic and distributional checklist of all the species of *Eustenogaster* are provided.

(**Fuki Saito-Morooka, Hari Nugroho, Alan Handru, and Jun-ichi Kojima**)

Keywords: distributional checklist, lectotype, new status, revised key, synonym

Treubia, 47(2): 99–110, December 2020

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CHARACTERS OF MITOCHONDRIAL DNA D-LOOP HYPERVARIABLE III FRAGMENTS OF INDONESIAN RHINOCEROS HORNBILL (*BUCEROS RHINOCEROS*) (AVES: BUCEROTIDAE)

Jarulis*[1], Choirul Muslim[1], Dedy Duryadi Solihin[2], Ani Mardiastuti[3], and Lilik Budi Prasetyo[3]

[1]Department of Biology, Faculty of Mathematics and Science, University of Bengkulu
[2]Department of Biology, Faculty of Mathematics and Science, IPB University
[3]Department of Conservation of Forest Resources and Ecotourism, Faculty of Forestry, IPB University
*Corresponding author: jarulis@unib.ac.id

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ABSTRACT

The Rhinoceros Hornbill (*Buceros rhinoceros*) genetic characteristics consist of nucleotide polymorphisms, haplotypes, genetic distances, and relationships which are important for their conservation effort in Indonesia. We sequenced mitochondrial DNA D-loop hypervariable III fragments from five Rhinoceros Hornbill individuals at Safari Park Indonesia I and Ragunan Zoo, which were isolated using Dneasy® Blood and Tissue Kit Spin-Column Protocol, Qiagen. D-loop fragment replication was done by PCR technique using DLBuce_F (5'-TGGCCTTTCTCCAAGGTCTA-3') and DLBuce_R (5'-TGAAGG AGT TCATGGGCTTAG-3') primer. Thirty SNP sites were found in 788 bp D-loop sequences of five Rhinoceros Hornbill individuals and each individual had a different haplotype. The average genetic distance between individuals was 3.09% and all individuals were categorized into two groups (Group I: EC6TS, EC1RG, EC2TS and Group II: EC9TS, EC10TS) with a genetic distance of 3.99%. This result indicated that the two groups were distinct subspecies. The genetic distance between Indonesian and Thai Rhinoceros Hornbills was 10.76%. Five Indonesian Rhinoceros Hornbill individuals at Safari Park Indonesia I and Ragunan Zoo probably came from different populations, ancestors, and two different islands. This study can be of use for management consideration in captive breeding effort at both zoos. The D-loop sequence obtained is a useful character to distinguish three Rhinoceros Hornbill subspecies in Indonesia.

Keywords: Bucerotidae, control region, phylogenetic, Rhinoceros Hornbill conservation, zoo

ABSTRAK

Karakter genetik enggang cula (*Buceros rhinoceros*) yang terdiri atas polimorfisme nukleotida, substitusi, haplotipe, jarak genetik, dan filogeni adalah data penting untuk konservasinya di Indonesia. Kami telah melakukan sekuensing fragmen hipervariabel III D-loop DNA mitokondria dari lima individu enggang cula asal Taman Safari Indonesia I dan Taman Margasatwa Ragunan, yang diisolasi menggunakan Spin-Column Protocol Dneasy® Blood and Tissue Kit Qiagen. Replikasi fragmen D-loop dilakukan dengan teknik PCR menggunakan primer DLBuce_F (5'-TGGCCTTTCTCCAAGGTCTA-3') dan DLBuce_R (5'-TGAAGGAGT TCATGGGCTTAG-3'). Tiga puluh situs SNP ditemukan pada 788 pb sekuen D-loop lima individu enggang cula dan tiap individu memiliki haplotipe berbeda. Jarak genetik rata-rata antar individu 3,09% dan semua individu terbagi menjadi dua group (Group I; EC6TS, EC1RG, EC2TS dan Group II; EC9TS, EC10TS) dengan jarak genetik 3,99%. Hasil ini menunjukkan bahwa kedua grup tersebut merupakan sub spesies berbeda. Jarak genetik antara enggang cula Indonesia dan Thailand 10,76%. Lima individu enggang cula Indonesia yang terdapat di Taman Safari Indonesia I dan Taman Margasatwa Ragunan diduga berasal dari populasi, nenek moyang, dan dua pulau berbeda. Informasi ini dapat digunakan untuk pertimbangan dalam usaha penangkarannya di kedua tempat tersebut. Sekuen D-loop yang diperoleh bermanfaat untuk membedakan tiga subspesies enggang cula di Indonesia.

Kata kunci: Bucerotidae, daerah kontrol, kekerabatan, konservasi *Buceros rhinoceros*, kebun binatang

INTRODUCTION

In Indonesia, Rhinoceros Hornbill (*Buceros rhinoceros* Linnaeus, 1758) only exists in Sumatra, Kalimantan, and Java. Based on its morphological characteristics, the species has been split into three subspecies, namely *Buceros rhinoceros rhinoceros* (Sumatra), *B. r. borneoensis* (Borneo), and *B. r. silvestris* (Java) (MacKinnon et al., 2010; Poonswad et al., 2013). However, those three subspecies are difficult to distinguish due to their morphological similarities. Therefore, genetic information needs to be analyzed to identify the differences between the subspecies.

The population of Rhinoceros Hornbill in its natural habitat has been decreasing until now. The two main factors causing the decrease are habitat loss and poaching. This species becomes the target of poaching in Indonesia to be used as accessories for traditional art and food ingredient (Bennett et al., 1997). In West Kalimantan Province, the feather of Rhinoceros Hornbill has been used as clothes and accessories for traditional dance performance (Kemp, 1995).

Conservation action to save Rhinoceros Hornbill from extinction due to loss of habitat and poaching is urgently needed. One of the efforts is by identifying its genetic characteristics (nucleotide polymorphisms, substitutions, haplotypes, genetic distances, and phylogeny) based on mitochondrial DNA (mtDNA) D-loop fragment. D-loop area is the non-coding area located between tRN^{Pro} and $tRNA^{Phe}$ (Desjardins & Morais, 1990), approximately 900 bp containing initial replication from heavy strand (Oμ) of mitochondrial DNA. It has a rapid evolution (Solihin, 1994), does not have coding for structural genes but mostly contains regulating element of mtDNA replication and transcription, regulates the genetic expression of mtDNA as well as has a high substitution rate, the highest among all genome. Therefore, it is suitable for testing the phylogeny within a species.

D-loop area is divided into three domains: domains I and III contain a high nucleotide variability, and domain II which is eternal in vertebrates (Randi & Lucchini, 1998). However, the initial section of D-loop domain I *Alectoris* (Phasianidae) at the side after $tRNA^{Glu}$, is evolving slower and having similar motifs with DNA sequence in mammals known as ETAS (Extended Termination Associated Sequences) I and ETAS II which can form a stable secondary structure (Sbisa` et al., 1997). The second section of domain I contains hypervariable area with two copies of tandemly repeated sequence DNA in other species from Anseriformes and Galliformes (Quinn & Wilson, 1993). The D-loop fragment is a non-coding region that lies between tRNPro and tRNAPhe (Desjardins & Morais, 1990), the size of approximately 900 bp which contains the start of the replication of heavy (Oμ) strands of mtDNA. Rapid evolution and has no coding for structural genes but mostly contains elements that regulate mtDNA replication, transcription, and mtDNA genetic expression regulators and

has a high mutation rate, even the highest in the entire genome, and it is very suitable for testing kinship in one species (Solihin, 1994).

The objectives of this research are 1) to analyze genetic characteristics consisting of nucleotide polymorphisms, (2) to describe nucleotide sites that undergo a mutation, the number and diversity of haplotype, and (3) to analyze genetic distances and relationships between individuals and populations of Rhinoceros Hornbill in Indonesia.

MATERIALS AND METHODS

Sampling

The research was conducted from May to October 2019. Five blood samples (0.5-1.0 ml) of Rhinoceros Hornbill were taken through ulnar vein and preserved with ethanol (Table 1). Blood samples from living specimens were collected from Taman Safari Indonesia (Safari Park Indonesia) I and Ragunan Zoo based on the approval document of animal ethics committee of IPB University No. 39-2106/2016. Molecular analysis was conducted in Molecular Biology Laboratory, Department of Biology, Faculty of Mathematics and Science, University of Bengkulu.

DNA isolation

The blood sample (15-25 mg) was washed for 3-5 times using buffer Tris-EDTA (low TE). Total DNA isolation was performed using Kit Dneasy® Blood and Tissue Kit Cat. No. 69504 (50) based on the procedure of *Spin-Column Protocol* Qiagen with modification. DNA quality resulted from the isolation was observed on agarose gel 1.2% using electrophoresis, and then stored in the freezer at -20°C before amplification.

DNA amplification and sequencing

Replication of target DNA in mitochondrial DNA D-loop fragment was performed through amplification using Polymerase Chain Reaction (PCR) technique. The primer was designed with Primer3 (http://bio-info.ut.ee/primer3-0.4.0/primer3) program via online based

Table 1. Total blood samples of rhinoceros hornbill analyzed

Sample No.	Sample Code	Ring Number /Name/Microchip	Sampling Site
1	EC2TS	TSIBGR17G044	Safari Park Indonesia I
2	EC6TS	TSIBGR18G036	Safari Park Indonesia I
3	EC9TS	'Loly'	Safari Park Indonesia I
4	EC10TS	'Yoan'	Safari Park Indonesia I
5	EC1RG	985121018297670	Ragunan Zoo

on alignment sequencing of D-loop area *Tockus erythrorhynchus* (GenBank access number AY027927). The primer sequences were DLBuceF (5'- TGGCCTTTCTCCAAGGTCTA -3') and DLBuceR (5'- TGAAGGAGTTCATGG GCTTAG -3') with the product size of 722 bp.

The mixture of PCR reaction (25 µl) consisted of 2-5 µl DNA template, for each 1.0 µl primer forward and reverse, 6.8 µl ddH_2O, 5.0 µl 5x buffer Qs, 5.0 µl 5x enhancer Qs, 1.0 µl dNTP, and 0.2 µl taq polymerase. The program of PCR machine for the amplification was as follows: pre-denaturation temperature 95^{o}C for 5 minutes, denaturation 94^{o}C for 1 minute, annealing 58^{o}C for 45 minutes, elongation 72^{o}C for 1 minute, extension 72^{o}C for 6 minutes, and cooling down 4^{o}C for 10 minutes. The cycle stage of denaturation-elongation was 35 times. DNA (3.0 µl) from the PCR result was visualized on agarose gel 1.2% with electrophoresis tools (Sambrook, 1989), and the result was taken with UV trans-illuminator (λ=300 nm). PCR product with light ribbon was sent to First Base Malaysia company for sequencing.

Data analysis

Nucleotide sequences (forward and reverse) of five Rhinoceros Hornbills were aligned using Clustal W program MEGA 6.0 (Tamura et al., 2011). BIOEDIT software version 7.0.9 (Hall, 1999) was used to edit the sequence of D-loop fragment and visualize the electrogram and its nucleotide base. The sequence of D-loop fragment of each individual was aligned with GenBank D-loop fragment through the Basic Local Alignment Search Tool-nucleotide (BLASTn) to see the similarities of the samples. The genetic distance among Rhinoceros Hornbill individuals was calculated using Kimura 2-parameter (K2P) model (Kimura, 1980).

The phylogeny tree was reconstructed using Neighbor-Joining (NJ) model K2P with 1000 times bootstraps (Tamura et al., 2011). One sequence of D-loop fragment, *Buceros rhinoceros* (GenBank access number GU560192), was downloaded from GenBank. *Tockus erythrorhynchus* was used as an outgroup species. The next step was analyzing the genetic characteristics of Rhinoceros Hornbill consisting of nucleotide polymorphism, substitution, haplotype, genetic distance, and constructing NJ tree. The number of haplotype was calculated using DnaSP program ver. 5.10 (Librado & Rozas, 2009). The median joining network of Rhinoceros Hornbill inter-individual haplotype was analyzed using Phylogenetic Network Software ver. 5 (Bandelt et al., 1999).

RESULTS

Characteristics and BLAST of D-loop fragment

The genetic characteristics of Rhinoceros Hornbill based on D-loop fragment are presented in Table 2. The length of nucleotide sequence of D-loop fragment of Rhinoceros Hornbill obtained was 788 bp. The result of five-fold alignment of mtDNA D-loop fragment

of Rhinoceros Hornbill demonstrated that the total varied nucleotide (varied sites and parsimony sites) was much higher (9.13%) than the singleton (3.8%). The highest composition of nucleotide base composing D-loop fragment sequence in all individuals was Adenin (38,4%) and the lowest was Guanin (11,7%). Base composition of GC (35.7%) for all individuals was smaller than AT base (64.3%). The sequence of nucleotide obtained was included in hypervariable area (domain III) located between site numbers 545 and 1572 of the intact sequence of *Aceros waldeni* D-loop fragment (GenBank access number NC015085).

The identification result using BLASTn showed that D-loop sequence of Rhinoceros Hornbill in GenBank was still limited (Table 3). The sequence of D-loop fragment of Rhinoceros Hornbill originated from Indonesia had an identity value of 87% compared to the Rhinoceros Hornbill from Thailand and query cover 98%.

Single nucleotide polymorphism

The number of specific site, Single Nucleotide Polymorphism (SNP) mtDNA D-loop fragment is presented in Table 4. The number of SNP D-loop fragment (788 bp) of five Indonesian Rhinoceros Hornbill was located in 30 sites. Thirty sites of SNP were between numbers 124 and 784. SNP was located in D-loop fragment hypervariable III area. The number of SNP suggested that Indonesia Rhinoceros Hornbill had specific sites that separated it from other Bucerotidae types in the world.

Table 2. Characterization of nucleotide sequence of Indonesian rhinoceros hornbill D-loop fragment with length (788 bp; n=5)

Characteristics	Rhinoceros Hornbill
Total types and individuals	1 and 5
Conservative sites (%)	737/788 (93.52)
Varied sites (%)	51/788 (6.47)
Parsimony sites* (%)	21/788 (2.66)
Singleton sites** (%)	30/788 (3.80)
Thymine (T) Percentage	25.8
Cytosine (C) Percentage	24.0
Adenosine (A) Percentage	38.4
Guanine (G) Percentage	11.7

Description: *Parsimony sites: found minimal two types of nucleotide, each type of nucleotide owned by minimal 2 sequences. **Singleton sites: different nucleotide only found in one sequence.

Table 3. Results of BLAST nucleotide sequence of rhinoceros hornbill D-loop fragment (788 bp)

Sample Code	Total Score	Query Cover (%)	Identity (%)	Species	GenBank Access Code
EC2TS	870	98	87	*Buceros rhinoceros*	GU560192
EC6TS	837	98	87	*Buceros rhinoceros*	GU560192
EC9TS	859	98	87	*Buceros rhinoceros*	GU560192
EC10TS	881	98	87	*Buceros rhinoceros*	GU560192
EC1RG	821	98	86	*Buceros rhinoceros*	GU560192

Table 4. Single nucleotide polymorphism in the sequence of Indonesian rhinoceros hornbill D-loop fragment (788 bp)

Species	Sample Code	Nucleotide Sequence Number																													
		1	1	1	1	2	2	3	3	4	4	4	4	4	5	5	5	5	6	6	6	6	7	7	7	7	7	7	7	7	7
		2	3	6	8	1	7	6	6	0	0	4	5	6	1	5	6	9	3	3	5	6	0	2	2	3	4	5	6	6	8
		4	5	2	9	3	3	0	1	1	2	4	8	7	9	4	5	2	5	6	7	7	5	1	2	5	9	0	1	4	4
Tockus erythro-rhynchus	GenBank Access No. AY027927	C	G	A	T	A	T	T	A	T	A	-	A	A	A	A	G	C	C	A	A	C	T	G	C	T	C	A	C	C	-
Buceros rhinoceros	EC6TS	T	C	.	.	T	.	A	.	.	.	C	.	.	C	G	A	.	A	.	.	.	C	.	.	A	T	G	.	T	C
Buceros rhinoceros	EC1RG	G	A	C	G	G	C	A	.	G	.	C	G	C	.	G	A	.	G	T	.	T	A	C	.	G	T	G	T	T	T
Buceros rhinoceros	EC2TS	T	A	C	.	G	C	A	.	.	T	C	.	.	.	G	A	.	A	.	.	.	A	.	.	G	T	G	T	A	T
Buceros rhinoceros	EC9TS	T	A	C	.	G	C	C	C	.	.	T	.	.	.	G	C	G	A	.	T	.	A	.	.	G	T	G	T	T	T
Buceros rhinoceros	EC10TS	T	A	C	.	G	C	A	.	.	.	C	.	.	.	T	A	.	A	.	.	.	A	.	T	G	G	.	T	T	T

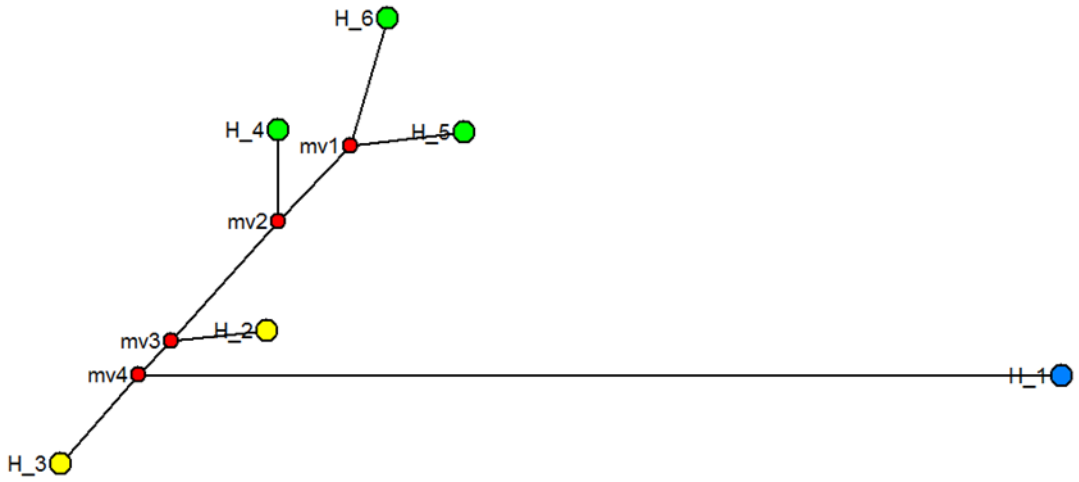
Description: Number one (1) of rhinoceros hornbill D-loop fragment nucleotide sequence is equivalent with number 15 *Tockus erythrorhynchus* (GenBank Access No. AY027927).

Haplotype and median joining network

The results of haplotype analysis showed that the five Rhinoceros Hornbill samples have different haplotypes (Table 5). Haplotype one was the Rhinoceros Hornbill from Thailand. Haplotypes two and three were close to each other and quite far from haplotypes four, five, and six based on the analysis using median joining network (Figure 1) with the diversity value of 1.0. All haplotypes of Rhinoceros Hornbill in Indonesia were separated quite far from haplotype one. This indicates that five Rhinoceros Hornbill individuals in Safari Park Indonesia I and Ragunan Zoo were originated from different ancestors and each Rhinoceros Hornbill has different ancestors. Moreover, Rhinoceros Hornbills at both zoos were from different populations and different descendants. This genetic information can be further used in the breeding program for Rhinoceros Hornbills in both zoos.

Table 5. Total and haplotype diversity of five rhinoceros hornbill in Indonesia based on D-loop fragment of 788 bp (n=5)

Haplotype Number	Total Individuals	Sample Code	Haplotype Diversity (Hd)
Haplotype 1	1	GenBank Acc. No. GU560192	1.0
Haplotype 2	1	EC9TS	
Haplotype 3	1	EC10TS	
Haplotype 4	1	EC2TS	
Haplotype 5	1	EC6TS	
Haplotype 6	1	EC1RG	



Description:
Buceros rhinoceros (GenBank Access Number GU560192)
EC9TS and EC10TS
EC1RG, EC2TS and EC6TS

Figure 1. Median Joining Network of Indonesian rhinoceros hornbill based on D-loop fragment with length 788 bp.

Genetic distance and phylogeny

The genetic distance among Rhinoceros Hornbill individuals depicts their phylogeny (Table 6). The genetic distance among individuals ranged from 0.0141 (1.41%) to 0.0485 (4.85%) with mean of 0.0309 (3.09%). The genetic distance between Indonesian Rhinoceros Hornbills and those from Thailand was 0.1076 (10.76%).

The reconstruction of phylogeny tree using NJ model showed that the five Indonesian Rhinoceros Hornbill individuals in Safari Park Indonesia I and Ragunan Zoo were divided into two groups (Fig. 2). Group 1 consisted of three individuals (EC6TS, EC1RG, EC2TS)

Table 6. Pairwise distance among Indonesian rhinoceros hornbill individuals

Sample	EC6TS	EC1RG	EC2TS	EC9TS	EC10TS	*B. rhinoceros* (GU560192)	*Tockus erythrorhynchus (AY027927)*
EC6TS							
EC1RG	0.0271						
EC2TS	0.0154	0.0219					
EC9TS	0.0418	0.0485	0.0337				
EC10TS	0.0404	0.0444	0.0324	0.0141			
B. rhinoceros (GU560192)	0.1120	0.1180	0.1032	0.1047	0.1003		
Tockus erythrorhynchus (AY027927)	0.2494	0.2602	0.2424	0.2283	0.2266	0.1785	

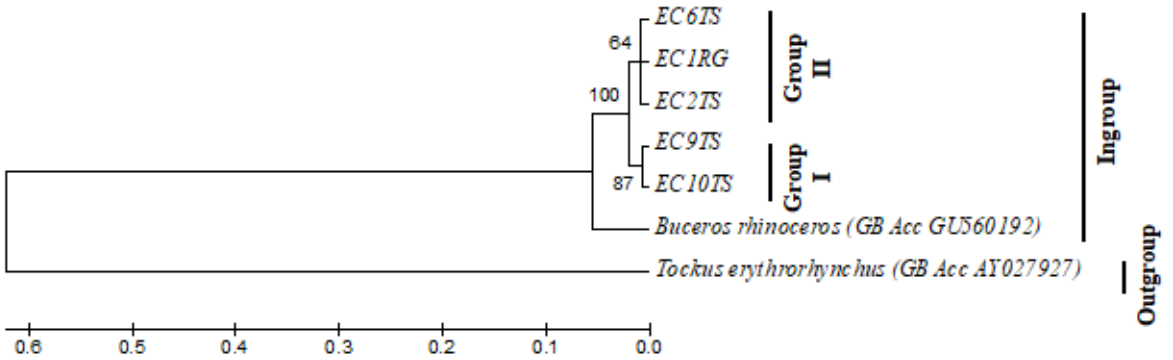


Figure 2. Neighbor-Joining phylogeny tree of Indonesian rhinoceros hornbill based on mtDNA D-loop fragment with length of 788 bp.

from Safari Park Indonesia I and Ragunan Zoo. Group 2 consisted of two individuals (EC9TS, EC10TS). The two groups were separated by genetic distance at 0.0399 (3.99%). This data suggested that five Rhinoceros Hornbill individuals in Safari Park Indonesia I and Ragunan Zoo were originated from different populations and could be assumed as different subspecies. However, the geographical origin of the two populations could not be determined since the D-loop fragment sequence data from the island of origin was unavailable.

DISCUSSION

Nucleotide polymorphism

The nucleotide polymorphism of five Indonesian Rhinoceros Hornbill samples from Safari Park Indonesia I and Ragunan Zoo was relatively high, with as many as 30 SNP sites from 788 bp between numbers 124 and 784 of III D-loop fragment hypervariable area. Adenine (38.4%) was nucleotide base with the highest composition while the lowest was

Guanine (11.7%), and nucleotide pair base GC (35.7%) for all individuals was smaller than AT (64.3%). Susanti et al. (2017) successfully amplified D-loop fragment of domestic water duck with product 718 bp using the primary DL-AnasPF (L56) and DL-AnasPR (H773). Widayanti (2006) found that the average composition of nucleotide *Tarsius bancanus* and *T. spectrum* was T 28.2%, C 25.8%, A 34.8%, and G 11.1% with the total AT of *T. bancanus and T. spectrum* was 63.1% and 62.9%, respectively. The composition of AT and GC in Acehnese cow was 63.31% and 36.66%, respectively (Abdullah, 2008). In D-loop area section of HVS-I Sumatran tiger, the research obtained the nucleotide diversity of 194 sites from the sequence of 567 bp with 12 specific nucleotide base sites (Faizah, 2008). In the sequence of hypervariable-1 D-loop length 397 bp in domesticated chicken, there were 76 polymorphic sites (variable site) consisting of 15 singleton variable sites and 61 parsimony informative sites (Zein & Sulandari, 2009).

The high nucleotide polymorphism or the differences in sequence of both sections of hypervariable of the non-coding region is used to distinguish between individuals of biological samples (Melton, 1999). D-loop is part of mtDNA that varies in nucleotide substitution, insertion or short deletion (indels) and having dynamic variable number tandem repeat (VNTRs) located in the hypervariative section and specific dominant (Fumagalli et al., 1996). D-loop area contains the most varied DNA sequence from the overall genome of animal's mtDNA and it is due to the high substitution rate around $0.075\text{-}0.165 \times 10^{-6}$ substitution/site/year (Sumida et al., 2000). D-loop fragment is a non-coding region with important role in the replication and transcription of mtDNA (Arif & Khan, 2009). The variation in mtDNA is due to higher substitution (five times higher) than other DNA region (Mannen et al., 2004; Pfeiffer et al., 2005).

Haplotype

Haplotype is collection of alleles or particular DNA sequences in a gen cluster related to chromosome that possibly inherited together (Nei, 1987). Each individual of the five samples of Indonesian Rhinoceros Hornbill has its own haplotype (Table 6) with haplotype diversity of 1.0. The results of median joining analysis (Fig. 1) showed that haplotypes two and three of Indonesian Rhinoceros Hornbill were close to each other but far from haplotypes four, five, and six. All haplotypes of Rhinoceros Hornbill in Indonesia were separated quite far from haplotype one. This suggests that five Rhinoceros Hornbill individuals in Safari Park Indonesia I and Ragunan Zoo were originated from different ancestors. According to this result, each Rhinoceros Hornbill has different ancestors. Therefore, Rhinoceros Hornbills at both zoos were from different populations and different descendants.

This genetic information can be used in the breeding program for Rhinoceros Hornbill at the above zoos. The variation in mitochondrial DNA can be explained by nucleotide polymorphism and haplotype (Yindee et al., 2010). Geographical isolation and reproduction encouraged new haplotype formation in the population (Coroian et al., 2015). The dominant haplotype is the center of main radiation in the population (Yuan et al., 2009). In addition, some haplotypes adjacent to the main haplotype show a specific nucleotide substitution. Palumbi (1994) said that population is separated due to the existence of individuals experiencing genetic diversity.

Genetic distance

The genetic distance between Indonesian Rhinoceros Hornbill and that from Thailand was 10.76%. With this rather far genetic distance, it is suspected that both samples are different species. The genetic distance among samples of Rhinoceros Hornbill describes their phylogeny (Table 6). The genetic distance among individuals ranged from 1.41% and 4.85% with a mean of 3.09%. This genetic distance was higher than genetic distance among individuals based on gene Cyt b (0.0-0.2%) and CO1 (0.7-0.11%) (Jarulis, 2019). In *Rhyticeros* spp., the genetic distance among individuals based on gene Cyt b was 0.1-0.7% (Jarulis et al., 2019). The genetic distance based on D-loop fragment in some mammals is lower than the result of this research. The genetic distance among individuals of *Tarsius bancanus* ranged at 1.0-3.0% and *T. spectrum* 0.0-1.0% (Widayanti, 2006), among individuals of Sumatran tiger 5.7-18.7% (Faizah, 2008), Indonesian cow 0.0-16.83% (Abdullah, 2008), and among individuals of Sumatran and Kalimantan rhinoceros was 2.9% (Zein et al., 2019).

Genetic distance between Group 1 (EC6TS, EC1RG, EC2TS) and Group 2 (EC9TS, EC10TS) was 3.99%. This number suggests that the two groups are different subspecies originating from two different islands. Unfortunately, we cannot identify the exact islands due to unclear information about the origin of the analyzed samples. Genetic distance between populations in *Buceros rhinoceros* found in this study was higher than the genetic distance between populations of Green Jungle Fowl originating from Jawa Tengah, Jawa Timur, Sumbawa, and Flores, which only ranged at 0.0 - 0.023 (Zein & Sulandari, 2008).

The analysis of D-loop fragment from the five Rhinoceros Hornbill individuals supports the assumption that three subspecies of Indonesian Rhinoceros Hornbill in Sumatra, Kalimantan, and Java could be separated into certain species, and they might have originated from two islands. However, this information requires further research on barcoding using genetic markers of COI, Cyt b, 12S rRNA, and 16S rRNA genes of mitochondrial DNA from the samples originating from the three islands.

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