

DNA Barcode of Mountain White-Eye *Zosterops montanus* (Aves: Zosteropidae) Based on Mitochondrial DNA Cytochrome Oxidase Sub Unit I

Jarulis^{1,*} Melisa Mayang Sari² Risky Hadi Wibowo¹

¹Department of Biology, Faculty of Mathematics and Natural Sciences, Bengkulu University, Indonesia

²Undergraduate Student, Department of Biology, Faculty of Mathematics and Natural Sciences, Bengkulu University, Indonesia

*Corresponding author. Email: jarulis@unib.ac.id

ABSTRACT

The study aimed to describe the genetic characteristic, single nucleotide polymorphism, genetic distance, and phylogenetic of mountain white-eye *Zosterops montanus* based on mitochondrial DNA CO1 gene and carried out in March-June 2020. A sample of the bird was taken from Pasar Minggu, Bengkulu city. The blood sample was taken from the pectoral vein, and the DNA genome was isolated using DNeasy® Blood and Tissue Kit based on Spin-Column Protocol procedure, Qiagen. The CO1 gene was amplified using a polymerase chain reaction technique, electrophoresis on 1.2% agarose gel, and visualized with a UV transilluminator. The results showed that the gene size of CO1 mitochondrial DNA from *Z. montanus* (n=5) is 751 bp, obtained conservative sites (C) 745 sites, variations (V) six sites, the parsimony of information (Pi) four sites, and singleton sites (S) two sites. The highest nucleotide base composition was cytosine (C) and the lowest guanine (G), while the highest nucleotide combined composition of adenine-thymine (AT) and the lowest guanine-cytosine (GC). We found six single nucleotide polymorphisms were evenly distributed between 9-732 sites. Average intraspecific genetic distance was 0.4%, interspecific genetic distance 4%, and between groups 1 and 2 separated from the outgroup (group 3) with a genetic distance of 12.5%.

Keywords: CO1 gene, DNA barcode, *Zosterops montanus*, Zosteropidae

1. INTRODUCTION

Indonesia is one of the ten countries with the largest biodiversity wealth in the world (mega diversity countries) after Colombia [1]. One of the diversity found in Indonesia is the type of bird. Indonesia has a very high diversity of bird species with some recorded birds, namely 1,589 species, 549 genera, 96 families, and 20 orders [2]. Unfortunately, now the number of birds in Indonesia tends to decrease. This can be caused by natural disasters, forest fires, poaching, habitat destruction, fragmentation, land-use change, illegal trade, and others.

Also, many people like to keep birds and participate in bird contests in various regions, causing positive and negative impacts. Some of the positive impacts, such as the development of a very profitable

bird business with bird breeding activities by the community, the development of the bird trade in bird markets, the growing trade in bird cages, bird feed, medicines, and bird vitamins, thus providing income for the community [3], but the negative impact can make bird populations decrease, and even cause birds to be endangered.

One of the birds that are threatened by its existence mainly due to hunting and illegal trade is the mountain spectacle bird. This bird is one of the species of the Zosteropidae family which has grayish-white feathers that surround the eyes, the body size is 11 cm, upper body color is olive green, light gray stomach color with brown sides, besides the iris. his eyes also appear pale [4]. Mountain White-eye bird is a warbler. The natural enemies of this bird are snakes and other animals, but the most dangerous ones are humans [5]. Mountain White-eye *Z. montanus* into

the IUCN red list of threatened species is at low risk (Least Concern) [6]. The increasing number of people, the more the needs, which can lead to reduced habitat or shelter, land-use change, rampant hunting, illegal trade, and others. Therefore, it is necessary to carry out genetic research related to the DNA of bird barcodes Mountain White-eye to be used as a database for further research.

The genetic marker used in studying, kinship, evolution, and genetic variation in various animal taxa is mitochondrial DNA [7]. Mitochondrial DNA has identical funding at the intra-species level of vertebrates because of its maternal inheritance pattern, which causes no recombination and high mutation rates. In the mitochondria, there are protein-coding genes, one of which is the CO1 gene or cytochrome oxidase subunit 1 [8]. CO1 gene has about 2000 base pairs defined as genes/markers barcode Internationally (The reference), but around 684-bp of the total length of these genes are believed to be used to identify animal species [9].

In the last few decades, the CO1 gene has been used as one of the founders of DNA because this CO1 gene has many advantages for studying genetic characteristics. After all, it has very few deletions and insertions in the sequence, and many parts are conserved, has a large number of copies. So, that it is easily amplified compared to genes originating from the nucleus so that it can be used as barcoding DNA in most species [10]. CO1 is also used as a study of phylogeny and population genetics [11], for the identification of Indonesian hornbills (Aves: Bucerotidae) [12] and genetic diversity in pheasants [13]. Research on the *Zosterops montanus* DNA barcode based on the mitochondrial DNA CO1 gene has never been carried out and the sequence data is not yet available in GenBank, so its status in the international classification is unclear. This study aims to obtain genetic characteristics, single nucleotide polymorphism, genetic distance, and phylogeny of the *Zosterops montanus* Mountain White-eye.

2. MATERIALS AND METHODS

This research was conducted in March-June 2020. *Z. montanus* samples were taken from Pasar Minggu, Bengkulu City, and further work was carried out at the Genetics and Biotechnology Laboratory, Basic Science Building, Biology Department, Faculty of Mathematics and Natural Sciences, Bengkulu University.

Mountain White-eye (*Z. montanus*) n = 5 were collected from traders who sell birds at the Sunday

market in Bengkulu city and originate from the Kepahiang district. Blood samples were taken using a syringe size of 1 ml as much as 0, 5-1 ml in the vein pectoral, and sampled direction is inserted into the vial containing EDTA for preservation, then stored in a freezer s hoot -20 °C.

Total DNA isolation was carried out using Dneasy® Blood Kit and Tissue Kit cat no 69504 (50) based on the modified Qiagen Spin-column Protocol procedure. The quality of the isolated DNA was observed in 1.2% agarose gel using electrophoresis, then stored in a freezer of -20°C before amplification.

CO1 gene amplification was carried out through an amplification process using the polymerase chain reaction (PCR) techniques. The primers used for CO1 amplification were designed using the tri primer program. The CO1 gene sequences used to design specific primers in this study came from the complete genome of mitochondrial DNA of the *Zosterops erythropleurus* type, which was found in GenBank with access code NC 027942 and the length of the CO1 sequence used was 1550 base pairs, namely ZCO1F (5'-TTCTGATTCTTTGGCCATCC-3') and ZCO1R (5'-GTTGGAAGGCTTTGCGT TTA-3').

The amplification reaction mixture using the PCR technique (total 25 µl) consisted of ddH₂O 9.8 µl, Qs buffer 5.0 µl, Qs enhancer 5.0 µl, dNTP 1.0 µl, 1.0 µl forward primer, reverse primer 1.0 µl, DNA template 2 µl, and Taq polymerase 0.2 µl. The condition of the PCR machine when amped is as follows; predenaturation temperature 95 °C for 5 minutes, denaturation 94 °C 1 minute, annealing 51 °C 45 seconds, elongation 72 °C 1minute, past elongation 72 °C 6 minutes, and cooling 4 °C 10 minutes. The number of cycles of the denaturation-elongation stage is 35 times. DNA (5.0 µl) PCR results were visualized on 1.2% agarose gel by electrophoresis and the results were photographed with UV transilluminator ($\lambda = 300$ nm). DNA band PCR products that match the target are sent to the company PT. First Base Malaysia for sequencing.

The sequence of nucleotides (forward and reverse) is edited by alignment using the Clustal W program MEGA 6.0 [14]. BIOEDIT software version 7.0.9 [15], was used for editing the CO1 gene sequence, and for visualization of its electrogram and the sequence of its nucleotide bases. Each individual's CO1 gene sequence was aligned with the CO1 gene in GenBank through the Barcode of Life Database (BOLD) to see the similarity of the samples tested. The genetic distance between individuals was calculated using the Kimura 2-parameter (K2P) method [16]. The phylogeny tree was reconstructed

Table 1. Number of samples used in data analysis.

No.	Species name	Sample code	Sample locations
1	<i>Zosterops montanus</i>	ZM1CO1	Bengkulu
2	<i>Z. montanus</i>	ZM2CO1	Bengkulu
3	<i>Z. montanus</i>	ZM3CO1	Bengkulu
4	<i>Z. montanus</i>	ZM4CO1	Bengkulu
5	<i>Z. montanus</i>	ZM5CO1	Bengkulu
6	<i>Z. lateralis</i>	Access code NC_029146	New Zealand
7	<i>Z. erythropleurus</i>	Access code NC_027942	China
8	<i>Z. japonicus</i>	Access code KT_601061	Japan
9	<i>Z. polioastrus</i>	Access code NC_032059	East Africa
10	<i>Z. abyssinicus</i>	Access code NC_032058	Southeast Kenya
11	<i>Z. borbonicus</i>	Access code MK_529728	French
12	<i>Yuhina diademata</i>	Access code NC_029462	China
13	<i>Y. diademata</i>	Access code KT_783535	China
14	<i>Y. gularis</i>	Access code MK_405666	China
15	<i>Y. nigrimenta</i>	Access code NC_040991	China
16	<i>Y. nigrimenta</i>	Access code MH_916608	China

Note: data number 6-16 are obtained from *GenBank*.

using the K2P Neighbor-Joining (NJ) model with 1000 replications [14]. The Mountain White-eye bird CO1 gene sequences contained in GenBank were downloaded and phylogeny tree constructions were included in the phylogeny tree reconstruction analysis.

3. RESULT AND DISCUSSION

3.1 Gene DNA Size and Species Identification

The result of electrophoresis in Figure 1 using a voltage of 50 Volt with a time of 45 minutes shows the size of DNA *Z. montanus* amplified by PCR technique according to the target planned at the time

of primary manufacture, namely 750 base pairs (bp). The PCR product is then followed by a sequencing process to obtain the nucleotide base sequence. Based on the sequencing results obtained throughout the length of the nucleotide sequence of individuals ($n = 5$) with forward ranging between 751-762 bp, while reverse ranges between 752-757 bp. After editing using Bioedit and MEGA 6, the sequence used in the data analysis was 751 bp. The length of the CO1 gene sequence obtained in this study is different from previous studies, which is longer than the Indonesian Hornbill CO1 gene (Aves: Bucerotidae) which measures 746 bp [12], Parrots are 567 bp [17] and Sea birds *Thalasseus* which measures 684 bp [18], but shorter than CO1 gene Cockatoo (Psittaciformes) measuring 807 bp [19]. The length of

the CO1 gene sequence in this study is still included in the length of the sequence that is often used in species barcodes.

The length of the CO1 gene sequence used to barcode species ranges between half of the total length of the CO1 gene and is located at the base of the sequence of all animal species [20]. Even short sequences (109-208 bp) are also very useful in identifying species [21]. The *Zosterops montanus* CO1 sequences ($n = 5$) of each individual (751 bp) were identified and matched with other *Zosterops* sequences available in GenBank in Table 2. CO1 gene identification is performed using the Barcode of Life Database (BOLD) System, online to see the percentage of the degree of similarity (similarity) CO1 gene of avian Mountain White-Eye (*Zosterops montanus*) available in GenBank.

The results of the BOLD System present the similarity value of all *Z. montanus* samples tested in Table 2. Three *Zosterops* spp species that were detected to have the highest similarity value with *Z. montanus* ($n=5$) were *Z. erythropleurus* with values ranging from 95.77 to 96.83% originating from China, *Z. lateralis* with values ranging from 94.18 - 95.24% came from New Zealand and *Z. polyogastrus* ranged from 93.65 to 94.71% from East Africa. The three identified species of *Zosterops* spp come from outside Indonesia. This indicates that the CO1 *Z. montanus* gene nucleotide sequence is not yet available in GenBank.

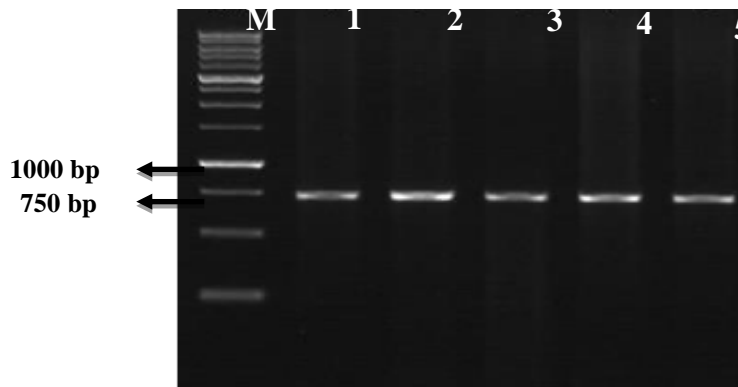


Figure 1. DNA band PCR result of CO1 *Z. montanus* gene. Information: M = Marker, 1 = ZM1CO1, 2 = ZM2CO1, 3 = ZM3CO1, 4 = ZM4CO1, 5 = ZM5CO1.

Table 2. Similarity of individual *Z. montanus* based on the results of the BOLD system of the CO1 gene length 751 bp.

Sample	BOLD System		Country of Origin (Access Code)
	Control Species	Similarity (%)	
<i>Z. montanus_1</i>	<i>Z. erythropleurus</i>	96.83	China (NC_027942)
	<i>Z. lateralis</i>	95.24	New Zealand (NC_029146)
	<i>Z. poliogastrus</i>	94.71	East Africa (NC_032059)
<i>Z. montanus_2</i>	<i>Z. erythropleurus</i>	96.83	China (NC_027942)
	<i>Z. lateralis</i>	95.24	New Zealand (NC_029146)
	<i>Z. poliogastrus</i>	94.71	East Africa (NC_032059)
<i>Z. montanus_3</i>	<i>Z. erythropleurus</i>	96.83	China (NC_027942)
	<i>Z. lateralis</i>	95.24	New Zealand (NC_029146)
	<i>Z. poliogastrus</i>	94.71	East Africa (NC_032059)
<i>Z. montanus_4</i>	<i>Z. erythropleurus</i>	95.77	China (NC_027942)
	<i>Z. lateralis</i>	94.18	New Zealand (NC_029146)
	<i>Z. poliogastrus</i>	93.65	East Africa (NC_032059)
<i>Z. montanus_5</i>	<i>Z. erythropleurus</i>	96.83	China (NC_027942)
	<i>Z. lateralis</i>	95.24	New Zealand (NC_029146)
	<i>Z. poliogastrus</i>	94.71	East Africa (NC_032059)

Note: control species data is obtained from the BOLD System linked to GenBank

Individual *Zosterops montanus* 1, 2, 3 and 5 had the highest similarity value with *Z. erythropleurus* 96.83 %, followed by *Z. lateralis* 95.24%, and *Z. poliogastrus* 94.71%, while *Z. montanus_4* had a value Similarity was different from the other 4 *Z. montanus* individuals, namely with the highest similarity value of *Z. erythropleurus* 95.77%, *Z. lateralis* 94.18% and *Z. poliogastrus* 93.65%. This difference can be caused because the *Z. montanus_4* has higher mutations or more nucleotide differences compared to other individuals. The lower the similarity value, the higher the level of nucleotide differences between *Z. montanus* and other *Zosterops* spp.

The similarity value between all tested individuals has a similarity value of less than 97.0%, so it can be stated that *Z. montanus* is a different species based on the CO1 gene with *Z. erythropleurus*, *Z. lateralis*, and *Z. poliogastrus*. According to Hebert *et al.* differences in similarity values between animal species have a threshold value of more than 3.0% [11].

3.2 Variation and Composition of Nucleotides

Based on the alignment of CO1 751 bp gene sequences, each individual *Z. montanus* (n = 5), obtained variations in nucleotides and nucleotide base compositions at the individual bird level. Mountain

White-Eye are shown in Table 3. The number of nucleotide sites conservatif (C) CO1 gene 745 sites (99.2%), variation sites (V) 6 sites (0.79%), information parsimony sites (Pi) 4 sites (0.53%), and singleton sites (S) 2 sites (0.26%).

The number of conservative sites, variations, parsimony, and singleton results of this study seemed different from the results of previous studies. Jarulis *et al*, found that the Aceros hornbill CO1 gene with length 746 bp had a conservative site of 737 sites (98.79%), varied 9 sites (1.2%), parsimony 0 sites (0%), singleton 9 sites (1, 2%). In the genus Rhyticeros conservative sites 730 sites (97.8%), variation 16 sites (2.14%), parsimony 11 sites (1.47%), singleton 5 sites (0.6%). Whereas in the Buceros genus the conservative sites were 727 sites (97.3%), the variation was 19 sites (2.54%), 2 sites parsimony (0.26%), 17 singleton sites (2.27%) [12]. Stated that the CO1 gene has advantages, namely conservative nucleotide sequences, slight deletions, insertions, and variations so that it is suitable for use as DNA barcoding [10].

The composition of each type of nucleotide base of 5 individual *Z. montanus* is shown in Table 3. The highest nucleotide base composition in all individuals was cytosine (C) which ranged between 28.1-28.6% and the lowest was Guanine (G) with a composition of 16.6%. According to Avise, protein-coding genes including CO1 are characterized by high cytosine and low guanine compositions [22]. The composition of the nucleotide bases from this study is directly proportional to several previous studies. The Simpidan bird CO1 gene with the highest composition of cytosine (C) was 29.4% and the lowest was Guanine (G) 17.3% [13]. CO1 of Indonesian hornbills, especially Anthracoceros, has the highest and lowest nucleotide base composition, namely Cytosine (C) 31.9% and Guanine (G) 15.6%, in Buceros namely Cytosine (C) 31.0% and the lowest is Guanine (G). 16.3%, and in Aceros, the

highest was cytosine (C) 29.6% and the lowest was Guanine (G) 16.4% [12].

The composition of the AT *Z. montanus* base pair was higher than that of GC which ranged from 54.8-55.3% and 44.7-45.2% respectively. According to Sueoka, in invertebrates, GC content generally ranges from 40–45% [23]. The composition of the two base pairs from this study is in line with the results of Astuti *et al*. with a composition of 53.5% AT and 46.5% GC of the Simpidan (Lophura) bird's CO1 gene [13]. Jarulis *et al*. with the percentage composition of the nucleotide base pairs AT and GC in Hornbills (Aves: Bucerotidae), respectively 52.8% and 47.2% [12]. Astuti and Sulandari found the base composition of AT and GC in cockatoos 51.9% and 48.2%, respectively [19].

The results of the composition of the nucleotide base pairs are inversely proportional to the research of, with a low AT composition with a value of 48.9%, while GC is higher with a value of 51.1% in eagles [24]. The composition of the nucleotide bases differs from one another can be caused by gene mutations. According to Karmana, the gene mutation is a difference that occurs in the DNA structure and the locus that does not change [25]. The difference in the number of nitrogen bases can cause the formation of different genes.

3.3 Single Nucleotide Polymorphism (SNP)

The results of alignment of nucleotide sequences using the CO1 gene between *Z. montanus* individuals showed the presence of single nucleotide polymorphism (SNP) as a differentiator between *Z. montanus* individuals as shown in Table 4. Based on tracking the number of single nucleotide polymorphisms, 6 specific sites were obtained from 5 individuals studied. Individual 1 has one specific site, namely number 474, individual 2 has three specifics

Table 3. Conservative, varied sites and base composition of individual *Z. montanus* CO1 genes nucleotides with 751 bp.

Sample	C	Vi	Pi	S	Composition of Nucleotides (%)			
					A	T	G.	C
<i>Zosterops montanus</i> _1					27.6	27.7	16.6	28.1
<i>Zosterops montanus</i> _2					27.4	27.3	16.6	28.6
<i>Zosterops montanus</i> _3	745	6	4	2	27.6	27.7	16.6	28.1
<i>Zosterops montanus</i> _4					27.4	27.4	16.6	28.5
<i>Zosterops montanus</i> _5					27.4	27.4	16.6	28.5

Note: C = conservative site, Vi = variable site, Pi = information parsimony site , S = Singleton, A = Adenine , T = Thymine, G = Guanine and C = Cytosine.

Table 4. SNPs between individuals of *Z. montanus* based on the CO1 gene with a length of 751 bp.

Individual (code)	Nucleotide Sites					
	9	10	198	474	513	732
<i>Zosterops erythropleurus</i> (NC_027942)	C	T	T	C	T	T
<i>Zosterops montanus</i> (1)	.	.	.	A	.	.
<i>Zosterops montanus</i> (2)	.	.	C	.	C	C
<i>Zosterops montanus</i> (3)
<i>Zosterops montanus</i> (4)	T	C	C	A	C	.
<i>Zosterops montanus</i> (5)	.	.	C	.	.	C

Note: Figures under the name situs nucleotides are site IDs for each site based on the alignment of the gene CO1 length of 751 bp.

sites, namely, at numbers 198, 513, and 735, individual 4 has five specific sites, namely at numbers 9, 10, 198, 474, and 513, while individual 5 has two sites specific numbers, namely the numbers 513 and 732.

SNP sites were evenly distributed along the CO1 gene sequence, namely from sites number 9 to 735 with the length of the CO1 sequence 751 bp. The results of this study are in line with Jarulis *et al* which received NSP between sequences number 84 and 737 on seven types of Indonesian hornbills [12]. The SNP sites obtained were genetic markers (species barcode) of *Zosterops montanus* and other types. So that it can be used to genetically identify *Zosterops*. Hebert *et al*, showed that the results of variations in the CO1 gene sequence can be used to distinguish types that are closely related to all taxa except the Cnidarians group [9]. Waugh (2007) said that each type has a specific nucleotide sequence with only slight differences in intraspecies [26].

3.4 Genetic Distance

Genetic Distance Results of Kimura 2-Parameter (K2P) analysis to determine the genetic distance between individuals of intraspecies *Z. montanus*, the genetic distance between *Z. montanus* and *Zosterops* in the same genus (GenBank data), and genetic distance between *Z. montanus* and *Yuhina* in the same family (GenBank data) shown in **Tables 5 and 6**. Based on the analysis results, it was found that the minimum value of intraspecies *Z. montanus* genetic distance was 0.0%, the maximum value was 0.7%

and the average was 0.4%. The genetic distance between *Z. montanus* individuals is by research on the type of cockatoo, which has a genetic distance ranging between 0.1% and 0.7% [17]. According to Waugh, the intraspecies genetic distance is usually less than 1% and rarely more than 2% [26]. The minimum value of the genetic distance between *Z. montanus* and *Zosterops* species outside Indonesia is 0.030 (3%), the maximum is 0.050 (5%) and the average is 0.040 (4%). Hebert *et al*. state that the difference in nucleotides and genetic distances of 3% between populations is a separate specie [27]. The minimum genetic distance between (*Zosterops* and *Yuhina*) of the Zosteropidae family is 0.117 (11.7%), the maximum is 0.140 (14%) and the average is 0.125 (12.5%). This study is in line with the research of Huang and Tu, which found that the average difference in the COI gene in the genus Ardeidae was 13.08% [28] and Yoo *et al*. in the Korean bird genera 8.2% [29]. Based on genetic distance, *Z. montanus* from Bengkulu has different species from 6 other *Zosterops* and 5 *Yuhina*.

Genetic distance is the level of difference in genes (nucleotide bases) in a species or population measured by numerical quantities [30]. Genetic distance can be used to determine the level of an animal's taxon at the species, genus, family, and higher levels. It can also describe the closeness between a species or in a population and is also used to see the closeness of genetic relationships between individuals [31].

Table 6. Genetic range mean intraspecies *Z. montanus*, interspecies *Zosterops* spp. and the intergenus Zosteropidae.

Genetic distance	Minimum (%)	Average (%)	Maximum (%)
Intraspecies <i>Z. montanus</i>	0.0	0.4	0.7
Intraspecies <i>Z. montanus</i> vs <i>Zosterops</i> spp.	3.0	4.0	5.0
<i>Zosterops</i> spp. vs <i>Yuhina</i> spp.	11.7	12.5	14.0

Table 5. Genetic distance matrix *Pairwise distance* intraspecies *Z. Montanus*, interspecies *Zozterops* , family intergenus *Zosteropidae*

<i>species</i>	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Z. montanus_1																
Z. montanus_3	0.004															
Z. montanus_2	0.001	0.005														
Z. montanus_4	0.005	0.007	0.004													
Z. montanus_5	0.005	0.007	0.004	0.000												
<i>Z. lateralis</i> (NC_029146)	0.034	0.039	0.033	0.037	0.037											
<i>Z. erythropleurus</i> (NC_027942)	0.033	0.034	0.031	0.030	0.030	0.037										
<i>Z. japonicus</i> (KT_601061)	0.047	0.048	0.046	0.044	0.044	0.037	0.033									
<i>Z. poliogastrus</i> (NC_032059)	0.037	0.041	0.036	0.037	0.037	0.039	0.033	0.044								
<i>Z. abyssinicus</i> (NC_032058)	0.041	0.046	0.040	0.042	0.042	0.037	0.036	0.046	0.012							
<i>Z. borbonicus</i> (MK_529728)	0.050	0.054	0.049	0.049	0.049	0.046	0.047	0.05	0.039	0.043						
<i>Yuhina diademata</i> (NC_029462)	0.135	0.140	0.136	0.138	0.138	0.143	0.136	0.138	0.137	0.139	0.130					
<i>Y. diademata</i> (KT_783535)	0.135	0.040	0.136	0.138	0.138	0.143	0.136	0.138	0.137	0.139	0.130	0.000				
<i>Y. gularis</i> (MK_405666)	0.128	0.132	0.126	0.131	0.131	0.122	0.116	0.128	0.117	0.119	0.12	0.127	0.127			
<i>Y. nigrimenta</i> (NC_040991)	0.118	0.123	0.117	0.122	0.122	0.114	0.112	0.105	0.106	0.110	0.117	0.114	0.114	0.085		
<i>Y. nigrimenta</i> (MH_916608)	0.118	0.123	0.117	0.122	0.122	0.114	0.112	0.105	0.106	0.110	0.117	0.114	0.114	0.085	0.000	

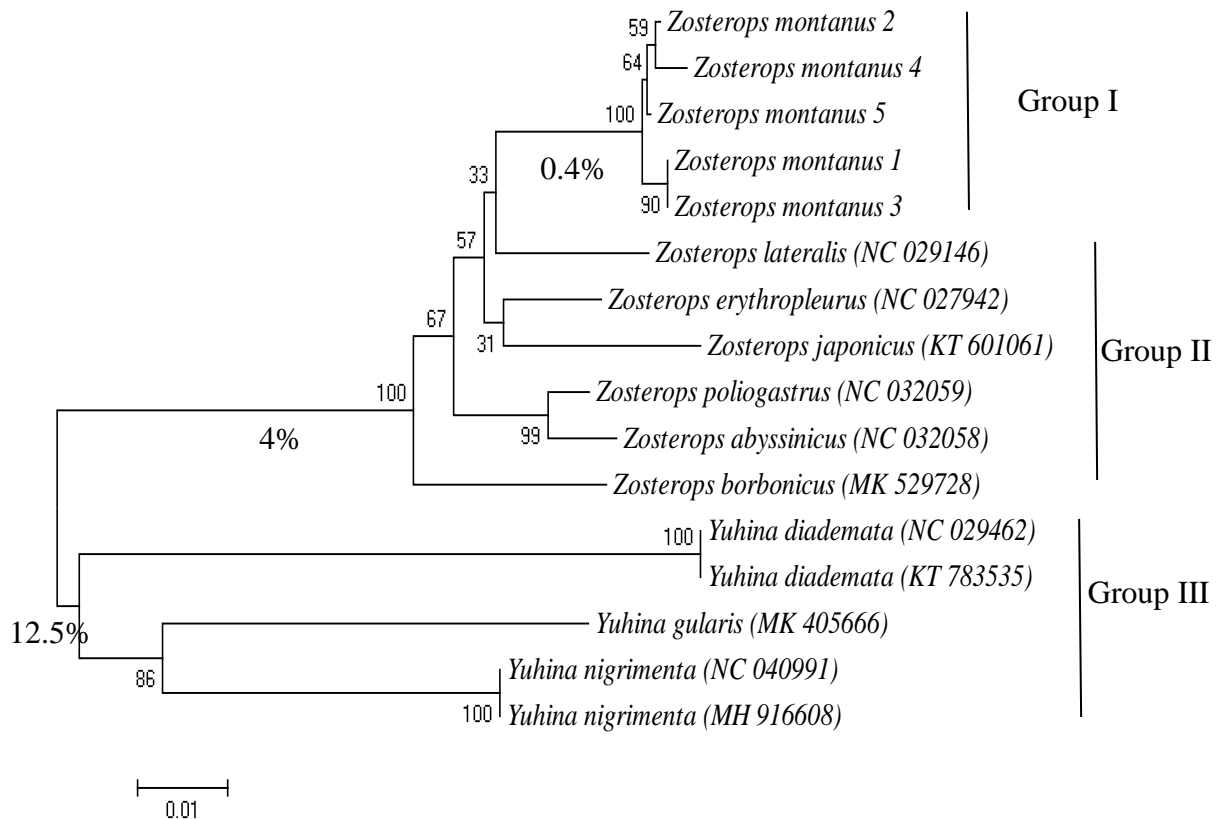


Figure 2. The phylogenetic tree was reconstructed using the K2P model and the *Neighbor-Joining* (NJ) *bootstrap* 1000 times based on the CO1 gene (751 bp).

3.5 Phylogenetic

A phylogenetic tree is a branch diagram depicting an arrangement of kinship relationships in a particular population or group [30]. The results of phylogenetic tree reconstruction using the K2P *Neighbor-Joining* (NJ) model with 1000 replications are shown in Figure 2. The results of phylogenetic tree analysis showed the separation of genetic distances into 3 groups. Group I consisted of 5 individuals, namely the *Z. montanus* bird studied, while *Z. lateralis*, *Z. erythropleurus*, *Z. japonicas*, *Z. poliogastrus*, *Z. abyssinicus*, *Z. borbonicus* formed group II and *Yuhina diademata*, *Y. gularis*, *Y nigrimenta* formed a separate group III.

The genetic distance of *Z. montanus* (n=5) 0.004 (0.4%) with a fairly high *bootstrap* value between 59 and 100. Group I and group II were separated by a genetic distance of 0.040 (4%), the *bootstrap* values ranged between 31 and 100, whereas between groups I and II separated with *outgroup* (group III) with a genetic

of 0.125 (12.5%). Differences in nucleotide and a 3% genetic distance between populations is a separate species [27]. The *bootstrap* value shows the closeness between one species and another, which is used to test how well the model data set we use is [32]. The CO1 gene is very effective as a DNA *barcode* due to the low variation between species while between species is high, especially in adjacent taxa [21].

Branch tree phylogenetic represents the relationship between the unit and describes the relationship hereditary, while the length of branch represents the number of evolutionary changes that occurred between two nodes [33]. Two sequences that have many similarities will be in one branch [32]. The diversity of CO1 gene sequences (648 bp) maintains a potential use for identification at the species level, and serves as a DNA *barcode* [8].

4. CONCLUSION

Based on the results of research on birds *Z. montanus* (n = 5) mitochondrial DNA CO1 gene size 751 bp, it was found that conservative sites (C) 745

sites (99.2%), variation (V) 6 sites (0.79%), parsimony information (Pi) 4 sites (0.53%), and *singleton* sites (S) 2 sites (0.26%). The highest composition of nucleotide bases was Cytosine (C) ranging from 28.1-28.6% and the lowest was Guanine (G) 16.6%, while the highest nucleotide composite composition was AT ranged from 54.8-55.3%, the lowest was GC. between 44.7-45.2%. There are six specific sites that are evenly distributed between sites 9-732 number. The average genetic distance of individual *Z. montanus* was 0.4% (group I), separated from other *Zosterops* spp. (Group II) with 4% genetic distance, group 1 and 2 were separated from outgroup (group III) with a genetic distance of 12.5 %.

ACKNOWLEDGMENTS

We would like to thank Drs. Muslim Choirul, SU., Ph.D. and Santi Nurul Kamilah, S.Si., M.Si. which has helped in the improvement of this article. We also express our appreciation to friends in the zoology laboratory who have helped during the research

REFERENCES

- [1] W. Novarino, Kobayashi, A. Salsabila, Jarulis, M.N Janra, Field Guide of Bird-Washing in Sumatra, National Library, 2008.
- [2] Sukmantoro, M. Irham, W. Novarino, F. Hasudungan, N. Kemp, M. Muchtar, Daftar Burung Indonesia No.2, Indonesia Ornithologists' Union, 2007. [In Bahasa Indonesia]
- [3] J. Iskandar, The dilemma between the hobby and business of bird trade and bird conservation, *Chimica et Natura Acta* 2 (3) (2014) 180–185.
- [4] J. Mackinnon, K. Philipps, B. Balen, Birds in Sumatra, Java, Bali and Kalimantan (Including Sabah, Sarawak and Brunei Darussalam), LIPI Biology, 2010. [In Bahasa Indonesia]
- [5] I. Yuwono, Pleci, PT AgroMedia Pustaka, 2013. [In Bahasa Indonesia]
- [6] IUCN, Bird Life International *Zosterops japonicus*, The IUCN Red List of Threatened Species 2019: e.T155158005A155636070, 2019.
- [7] T.D. Kocher, W.K. Thomas, A. Meyer, S.V. Edwards, S. Paabo, F.X. Villablanca, A.C. Wilson, Dynamics of Mitochondrial DNA Evolution in Animals: Amplification and Sequencing with Conserved Primers, *Proc. Natl. Acad. Sci. USA*, vol. 86, 1989: pp. 6196–6200.
- [8] P.D.N. Hebert, M.Y. Stoeckle, T.S. Zemplak, and C.M. Francis, Identification of birds through DNA barcodes, *PLoS Biol* 2(10) (2004) 312.
- [9] P.D.N. Hebert, S. Ratnasingham, J.R. DeWaard, Barcoding Animal Life: Cytochrome C Oxidase Subunit 1 Divergences Among Closely Related Species, *Proceedings of the Royal Society B: Biological Sciences*, vol. 270, 2003. DOI: <https://doi.org/10.1098/rsbl.2003.0025>.
- [10] M.S.A. Zein, D.M Prawiradilga, DNA Barcode Fauna Indonesia, Kencana Prenadamedia, 2013. [In Bahasa Indonesia]
- [11] M. Hajibabaei, G.A.C. Singer, P.D.N. Hebert, D.A. Hickey, DNA Barcoding: How it Complements Taxonomy, *Molecular Phylogenetics and Population Genetics, Trends in Genetics*, 2007, pp. 167–172. DOI: <https://doi.org/10.1016/j.tig.2007.02.001>.
- [12] Jarulis, D.D. Solihin, A. Mardiasuti, L.B. Prasetyo, DNA Barcode of Seven Indonesian Hornbills Species (Aves: Bucerotidae) Based on Mitochondrial DNA Cytochrome Oxidase Subunit I, *HAYATI Journal of Biosciences* 25(4) (2018) 178–187. DOI: <https://doi.org/10.4308/hjb.25.4.178>.
- [13] D. Astuti, M. Irham, H. Ashari, COI Gene Nucleotide Variations in Narrow Bird (Genus *Lophura*), *Indonesian Biology* 14(1) (2018) 103–108.
- [14] K. Tamura, G. Stecher, D. Peterson, A. Filipksi, S. Kumar, MEGA6 Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology dan Evolution* (2013) 1–5. DOI: <https://doi.org/10.1093/molbev/mst197>.
- [15] T.A. Hall, BioEdit: a User-Friendly Biological Sequence Alignment Editor dan Analysis Program for Windows 95/98NT, vol.41, *Nucleic Acids Symposium Series*, 1999 pp. 95–98.
- [16] M. Kimura, A Simple Method for Estimating Evolutionary Rates of Base Substitutions Through Comparative Studies of Nucleotide

- Sequences. vol. 16, Journal of Molecular Evolution, 1980, pp. 111–120.
- [17] P.F.M Goncalves, A.R Oliveira-Marques, T.E. Matsumoto, C.Y. Miyaki, DNA Barcoding Identifies Illegal Parrot Trade. *Journal of Heredity*, 106(1) (2015) 560–564. DOI: <https://doi.org/10.1093/jhered/esv035>.
- [18] M.A. Efe, E.S. Tavares, A.J. Baker, S.L. Bonatto, Multigene Phylogeny and DNA Barcoding Indicate that the Sdanwich Tern Complex (*Thalasseus sandvicensis*, Laridae, Sternini) Comprises Two Species, *Molecular Phylogenetics dan Evolution* 52(1) (2009) 263–267. DOI: <https://doi.org/10.1016/j.ympev.2009.03.00>.
- [19] D. Astuti, S. Sulandari, The DNA Sequence Performance of CO1 Gene in White Cockatoos (Cacatua, Psittaciformes), *Treubia* 37 (2010) pp. 1–14
- [20] S. Sammler, C. Bleidorn, R. Tiedemann, Full Mitochondrial Genome Sequences of Two Endemic Philippine Hornbill Species (Aves: Bucerotidae) Provide Evidence for Pervasive Mitochondrial DNA Recombination, *BMC Genomics* 2011, pp. 1–10. DOI: <https://doi.org/10.1186/1471-2164-12-35>.
- [21] M. Hajibabaei, M.A. Smith, D.H. Janzen, J.J. Rodriguez, J.B. Whitfield, and P.D.N. Hebert, A Minimalist Barcode Can Identify a Specimen whose DNA is Degraded, *Molecular Ecology Notes* 6(4) (2006) 959–964. DOI: <https://doi.org/10.1111/j.14718286.2006.01470.x>.
- [22] J.C. Avise, *Molecular Markers, Natural History and Evolution*. Chapman and Hall, 1994
- [23] N. Sueoka, On the Genetic Basis of Variation dan Heterogeneity of DNA Base Composition. *Genetics*, vol. 48 (1962) pp. 582–591.
- [24] M.S.A. Zein, DNA Barcode of the Eagle (Family Accipitridae) in Indonesia, *LIPI* 17(2) (2018) 165–173.
- [25] O. Karmana, *Biology*, Grafindo Media First, 2008.
- [26] J. Waugh, DNA Barcoding in Animal Species: Progress, Potential dan Pitfalls, *BioEssays* 29(2) (2007) pp. 188–197. DOI: <https://doi.org/10.1002/bies.20529>.
- [27] P.D.N. Hebert, A. Cywinska, S.L. Ball, J.R. DeWaard, Biological Identifications Through DNA barcodes, *The Royal Society* 270 (2003) pp. 313–321.
- [28] Z. Huang, F. Tu, DNA Barcoding dan Phylogeny of *Calidris* and *Tringa* (Aves: Scolopacidae), *Mitochondrial DNA* 28(4) (2016) 616–619. DOI: <https://doi.org/10.3109/24701394.2016.1155121>
- [29] H. Yoo, J. Eah, J. Kim, Y. Kim, M. Min, W. Paek, C. Kim, DNA Barcoding Korean Birds, *Molecules and Cells* 35(4) (2013) pp. 357. DOI: <https://doi.org/10.1007/s10059-013-3151-6>.
- [30] M. Nei, S. Kumar, *Molecular Evolution and Phylogenetics*, Oxford University Press, 2000.
- [31] Wirdateti, E. Indriana, Handayani, Analysis on Mitochondrial DNA Cytochrome Oxidase I (COI) Sequences of Indonesia Slow Lories (*Nycticebus* spp) as Marker to Improve Identification of Species, 12(1) (2016) 119–128. DOI: <https://doi.org/10.14203/jbi.v12i1.2322>.
- [32] I. Dharmayanti, *Molecular Phylogenetics: Taxonomic Methods of Organisms Based on the History of Evolution*, WARTAZOA 21(1) (2011) 1–9.
- [33] D. Graur, W. Li., *Fundamental of Molecular Evolution Second Edition*. Sinauer Associates Inc. Sunderland, (2010) DOI: <https://doi.org/10.1016/B978-1-4557-5134-1.00001-9>.