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Genetic Variation of Black Capped White Eye *Zosterops atricapilla* (Aves: Zosteropidae) Based on Mitochondrial DNA Cytochrome B Gene

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ABSTRACT
The purpose of this research was to explore the genetic variation of Black Capped White eye *Zosterops articapilla* using the mitochondrial DNA c*ytochrome* b gene. The blood sample was collected via pectoral vein of bird from Panorama markets, Bengkulu city. DNA genome were isolated and purified from the blood following DNeasy protocol® Blood and Tissue Kit cat no. 69504 (50), based on Qiagen's Spin-Column Protocol procedure. We used polymerase chain reaction machine for amplification DNA template with specific primer (ZCYTBF and ZCYTBR). The results showed that length of cyt b gene sequence (n=6) was 725 bp and had 717 bp (98.89%) conservative site. The average of intraspecific genetic distance was 0.04, interspecific 0.063, and between Indonesian and overseas (outgroup) was 0.138.

Keywords: *Cytb, Conservation, illegal trafficking, mtDNA, Zosteropidae*

1. INTRODUCTION

Indonesia has an area of approximately 1.9 million km^2 of land, with a total area of 8 million km^2. With this vast area, Indonesia has approximately 47 different ecosystems and is supported by Indonesia's location in the cross-distribution area of biodiversity from the continents of Asia and Australia and is a transitional area of Wallacea which allows Indonesia to have high endemic species and have unique characteristics and also island location, which are far apart resulting in high speciation resulting in high diversity [1].

One family of aves is Zosteropidae which has 12 genera, and the number of species is about 85 individuals, which are spread unevenly throughout the world. Approximately 50 of these species are located in the Indo-Australian region, their status in the IUCN of Eye glass birds is low risk (LC / least concern). However, the poaching and habitat destruction are a concern for the survival of birds. These conditions, being hunted and trafficked, it can endanger the survival of the bird [2].

According to [3], endangered animals have low levels of genetic variation. The effort that can be made to preserve a species from the threat of extinction is to conserve it ex situ or in situ. The molecular approach can be used as a way of genetic conservation where conservation of this type is a step to save genetic resources by using DNA markers. Therefore, knowledge of variations is required at the molecular level using molecular marker analysis and has been useful for conservation efforts using DNA markings [4].

2. MATERIALS AND METHOD

2.1. Sample Collections

Six individuals of Black Capped White Eye *Zosterops atricapilla* was collected from Panorama bird market, Bengkulu city (Table 1). Blood was taking out through the pectoral vein 0.5-1 ml using a 1,0 ml syringe. The blood was put into an EDTA bottle for preservation and stored in a freezer with a temperature of -20C.

2.2. DNA Isolation

Total DNA isolation was carried out using the Dneasy® Blood 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 -20^{0}C before amplification.

Table 1. Sample Data Analyzed and sample from Genbank

No	Sample	Sample code/ Accession Number	Region
1	*Zosterops atricapilla*	ZA1CYTB	Bengkulu
2	*Zosterops atricapilla*	ZA2CYTB	Bengkulu
3	*Zosterops atricapilla*	ZA3CYTB	Bengkulu
4	*Zosterops atricapilla*	ZA4CYTB	Bengkulu
5	*Zosterops atricapilla*	ZA5CYTB	Bengkulu
6	*Zosterops atricapilla*	ZA6CYTB	Bengkulu
7	*Zosterops atricapilla*	JN827239	Mainland Asia and Afrika
8	*Zosterops erythropleurus*	NC027942	China
9	*Zosterops abyssinicus*	NC032058	Southeast Kenya
10	*Zosterops bobonicus*	MK529728	French
11	*Zosterops japonicus*	KT601061	Japan
12	*Zosterops lateralis*	NC029146	Selandia Baru
13	*Zosterops poligastrus*	NC032059	East Africa
14	*Yuhina diademata*	NC029462	China
15	*Yuhina diademata*	KT783535	China
16	*Yuhina gularis*	MK405666	China
17	*Yuhina nigrimenta*	NC040991	China
18	*Yuhina nigrimenta*	MH916608	China

Sample in this study 1-6, and 7- 18 sample from the *Genbank*

2.3. PCR and Sequencing

Target DNA replication in cytochrome b gene is carried out through the amplification process using polymerase chain reaction (PCR) techniques. The primer used for Cytochrome b amplification is designed using the primary tree program available online. Sequence of Cytochrome b gene used to design specific primers in this study derived from the complete genome of mitochondrial DNA of the *Zosterops erythropleurus* type which collected from *GenBank* (accesion number NC 027942), namely ZCYTBF (5'-GACACCAACCTAGCCTTTGC-3') and ZCYBR (5'- AACTAGCACT GAG GCGGCTA-3'), and sequencing DNA send to PT. First Base Malaysia.

2.4. Data Analysis

The sequenced nucleotide data (forward and reverse) were edited by alignment using the Clustal W program MEGA 6.0 [5]. BIOEDIT software version 7.0.9 [6], is used for editing the Cytochrome b gene sequences, and visualization of its electrogram and the sequence of its nucleotide bases. The Cytochrome b gene sequences of each individual were aligned with the Cytochrome b genes that were obtained from GenBank through the basic local alignment search tool-nucleotide (BLASTn) to checking the similarity of the samples tested. The genetic distance between individuals was calculated using Kimura 2-Parameter (K2P) method [7]. The phylogeny tree was reconstructed using the K2P Neighbor-Joining (NJ) model with 1000 replications [5]. We use twelve of cytochrome b sequence form GenBank for analysis.

3. RESULTS AND DISCUSSION

3.1. PCR Product

Visualization results showed the presence of DNA bands in the six blood samples of *Zosterops atricapilla* (Figure 1). The length of the sequences obtained was different from previous studies. The research of [8] obtained the length of sequences in the Paridae, Remizidae, and Aegithalos families 948 bp. The research of [9] obtained the sequence length of the three Indonesian hornbill species of 849 bp, in [10] study using a sample of parrots the sequence length was 791 bp. Based on this description, it can be concluded that the length our Cyt b sequence was shorter than in previous studies.

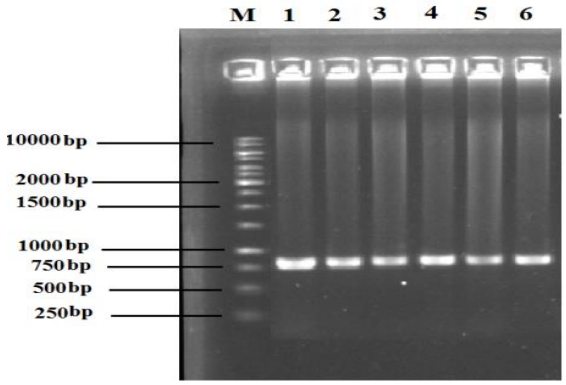

Figure 1. Visualization by gel documentation of *cyt* b in *agarose* 1.2%.

Table 2. BLASTn result of the Black Capped White Eye Cyt b gene 725 bp

Sample	BLASTn			Locality
	Species	Query cover (%)	Identity (%)	
Zosterops atricapilla	*Z. erythropleurus*	100	97,70	Beijing, China
	Z. japonicus	99	96,14	Japan
	Z. atricapilla	99	95,86	Daratan Asia dan Afrika
Zosterops atricapilla	*Z. erythropleurus*	99	96,42	Beijing, China
	Z. japonicus	99	95,86	Jepang
	Z. atricapilla	99	95,59	Mainland Asia dan Africa
Zosterops atricapilla	*Z. erythropleurus*	100	96,42	Beijing, China
	Z. japonicus	99	95,86	Japan
	Z. atricapilla	99	95,59	Mainland Asia dan Africa
Zosterops atricapilla	*Z. erythropleurus*	100	96,56	Beijing, China
	Z. japonicus	99	96,00	Japan
	Z. atricapilla	99	95,72	Mainland Asia dan Africa
Zosterops atricapilla	*Z. erythropleurus*	99	96,28	Beijing, China
	Z. japonicus	99	95,59	Japan
	Z. atricapilla	99	95,31	Mainland Asia dan Africa
Zosterops atricapilla	*Z. erythropleurus*	100	96,56	Beijing, China
	Z. japonicus	99	96,00	Japan
	Z. atricapilla	99	95,72	mainland Asia dan Afrika

3.2. Species Identification

Based on the results of BLASTn, we found that the highest identity of all samples (n = 6) was 97.70% and the lowest identity 95.31%. All the *Zosterops* spp. species identified in the Genbank data come from outside Indonesia, and there are one species of the same type but different places of collection, namely in the plains of Asia and Africa with the lowest identity value of 95.31% and the highest 95.72%. These data suggest that our *Zosterops atricapilla* Cyt b gene sequence are new data.

3.3. Nucleotide Variation and Composision

Variation and composition of nucleotides from intraspecies to the six samples of *Zosterops atricapilla* by the results of the Cyt b gene alignment in MEGA 6 application and assisted with Clustal W, has 717 of a conservative site (98.89%), 8 bp of variable site (1.10 %), parsimony 4 (0.55%) site, and singleton 4 (0.55%) site. Total sites varied were 12 sites (1.67%).

The composition of each type of nucleotide base of the six individuals of *Zosterops atricapilla* were quite varied (Table 3). The type of nucleotide that has the highest composition in the six species was cytosine with a value of 32.4 - 32.7% and the lowest was guanine with a value of 15.4 - 15.6%, this is by [10] that the composition of guanine has the composition smaller than the cytosine composition. The composition of the nucleotide base pairs of Adenine and Thymine is known to be above GC with AT values ranging from 51.7% - 52% and GC from 47.8% - 48.3%. In the study [9] the composition of AT was higher than GC and in research [10] the composition of AT and GC were 50.7% and 49.3% in the genus *Lophura* (Galliformes) respectively.

Table 3. Character and Nucleotide Composition of Zosterops atricapilla based on Cyt b Gene with length 725 bp.

Species	Conserved Site	Variable Site			Nucleotide Composision (%)			
		V	Pi	S	A	T	G	C
*Zosterops atricapilla*1	717	8	4	4	27.0	25.0	15.6	32.4
*Zosterops atricapilla*2					27.2	25.0	15.4	32.4
*Zosterops atricapilla*3					26.9	24.8	15.6	32.7
*Zosterops atricapilla*4					26.9	25.0	15.6	32.6
*Zosterops atricapilla*5					27.0	24.8	15.6	32.6
Zosterops atricapilla6					27.2	25.0	15.4	32.4

Note: V = Variable, Pi = Parsimoni, S = Singleton, A = Adenin,
T = Timin, G = Guanin, C = Sitosin

3.4. Nucleotide Polymorphism

The results of the alignment of the Cyt b gene sequence from the six individuals of *Zosterops atricapilla* showed a specific nucleotide or single nucleotide polymorphism (SNP) (Table 4). A total of 8 different nucleotide sites were found located between sites number 7 and 725. *Zosterops atricapilla* 5 was the individual that has the most specific nucleotides (five sites) compared to other. In the research of [9], a total of 17 different nucleotide sites were found, which are between sites number 41 and 671. Each species has a different length of nucleotide sites, in the research of [9], the types of hornbills studied had different nucleotide base sites, each type of species had slight differences in intraspecies in their specific nucleotide sequences [11]. Cyt b gene nucleotides can be used as genetic markers in explaining genetic diversity in flocks of chickens [12].

Table 4 SNP of Zosterops atricapilla based on Cyt B gene with length 725 bp

Individual	Sequence number							
	7	201	270	330	427	672	699	725
Zosterops erytropleurus	A	G	T	A	C	A	T	G
Zosterops atricapilla 1	.	.	.	.	.	.	.	.
Zosterops atricapilla 2	.	.	.	.	T	.	C	A
Zosterops atricapilla 3	C	.	C	.	.	.	.	.
Zosterops atricapilla 4	C	.	.	.	.	.	.	.
Zosterops atricapilla 5	.	A	.	G	.	G	C	A
Zosterops atricapilla 6	.	A	.	.	.	.	.	.

3.5. Genetic Distance

Genetic distances were calculated using the Kimura 2-Parameter method. Then the minimum value for group one is 0.01% and the highest 1%. Genetic distance in the same family has a minimum value of 3.4% and maximum 8.5%. A minimum value of genetic distance in the same genus was 1.3% and a maximum 15.4%. This genetic distance has the same tendency as previous studies, in the study of [8] used the Paridae and Remizidae families found the mean distance between species was 0.081 and 0.007, the mean distance between the genus was 0.077 and 0.005, and the distance between taxa was 0.115 and 0.009. In [10] study using parrots, the minimum distance ranged between 1.0% and 22.8%, and in the research of [9] the minimum genetic distance in the Indonesian hornbill species was obtained 4.80%, the maximum genetic distance is 5.70%, and the mean distance for group one is 0.04 (4%) in group two 6.3% and 13.8% in group three. in the research of [10] the genetic distance of the three Lophura individuals is 0.13%.

In GenBank, there are the same species which are then used as a reference to see the genetic distance of the two species (Figure 2). *Zosterops atricapilla* species with GenBank accession number JN827239, samples were taken from mainland Asia to Africa, while the sample used in the study was *Zosterops atricapilla* from Indonesia, and it can be seen that the genetic distance of the two samples was quite far apart but still in the same cluster. Factors affecting the genetic distance of a species are low, namely, the number of samples analyzed is small, the genetic diversity of birds taken as a small sample due to their small population, and the birds are endemic [13].

Table 5. Genetic distence average of intraspecies and interspecies of Z. atricapilla

Genetic Distance	Min.	Max.	Average
Interspesies *Z. Atricapilla*	0,001	0,010	0,004
Z. atricapilla vs interspecies *Zosterops*	0,034	0,085	0,060
Z. atricapilla vs *Yuhina*	0,013	0,154	0,132

The genetic distance is expressed close if the value ranges from 0.010 - 0.099%, expressed moderately when ranged from 0.100 - 0.990% and is expressed far in the value range of 1-2%, two individuals have genetic closeness if the resulting genetic distance value is not more than 0.1% [14].

Phylogeny analysis usually used to determine how the species was passed down during evolution. In the phylogenetic tree the rate of evolution of a species is demonstrated by horizontal lines and vertical lines on the phylogenetic tree indicating the genetic distance between species. Phylogenetic analysis can be used to determine the proximity and genetic relationship between one species and another [15], phylogenetic analysis using 18 samples, of which 6 main samples and 5 samples are based on genus, 5 samples by family and 1 sample as a reference and 1 sample of the same type of different origin all taken in GenBank. The result of phylogenic tree reconstruction was taken using the Neighbor Joining model with 1000 bostrap reps (Figure 2).

4. CONCLUSION

Based on the results of research on Genetic Variations in *Zosterops atricapilla* from Bengkulu, it proves that from the six samples of *Zosterops atricapilla* with interspecific variations, genetic distance, samplified using range e.g: 0.1%-1.0%, genetic distance in the same family samplified using range 3.4%-8.5%, genetic distance in the same genus with a minimum value of 1.3% and maximum 15.4%. The mean distance for group one was 0.4%, in group two was 6.0% and in group three was 13.2%.

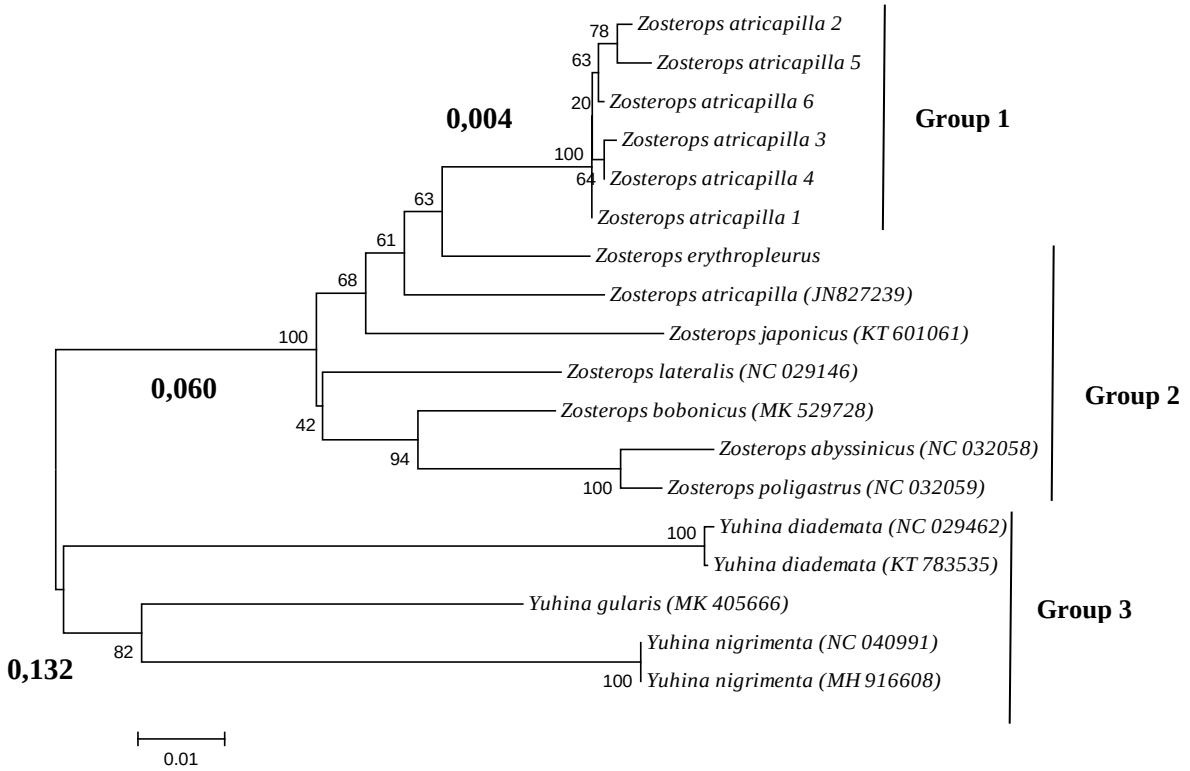

Figure 2. PhylogeneticTree of Neighbor Joining 6 individual *Zosterops atricapilla* construction with K2P modeling bootstrap 1000x basis on Cyt b gene mtDNA (725bp) .

REFERENCES

[1] W. Novarino, H. Kobayashi, A. Salsabila, Jarulis, N. Janra, Panduan lapangan pencincinan burung di Sumatera (Edisi Pert), Padang, Perpus takaan Nasional, 2008. [In Bahasa Indonesia]

[2] IUCN, *Zosterops atricapilla*, 8235, 2016.

[3] S.L. Ardern, D.M. Lambert, Is the Black Robin in genetic peril? Molecular Ecology 6(1) (1997) 21–28. https://doi.org/10.1046/j.1365294X.1997.00147.

[4] J.B. Whittier, D.M. Leslie, R.A. van Den Bussche, Genetic variation among Subspecies of least tern (Sternaantillarum): Implications for Conservation, Water birds 29(2) (2006) 176–184. https://doi.org/10.1675/15244695(2006)29[176:gvasol]2.0.co;2.

[5] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA 6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 1–5 (2013). DOI: https://doi.org/10.1093/molbev/mst197. [In Bahasa Indonesia]

[6] T.A. Hall, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98NT, Nucleic Acids Symposium Series 41 (1999) 95–98.

[7] M.A. Kimura, Simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, Journal of Molecular Evolution, 16 (1980) 111–120.

[8] C. Dai, K. Chen, R. Zhang, X. Yang, Z. Yin, H. Tian, F. Lei. Molecular phylogenetic analysis among species of Paridae, Remizidae and Aegithalos based on mtDNA sequences of COI and cyt b, Chinese Birds 1(2), (2010) 112–123. DOI: https://doi.org/10.5122/cbirds.2010.0003.

[9] Jarulis, D.D. Solihin, A. Mardiastuti, L.B. Prasetyo, Variasi interspesifik Julang (Aves:Bucerotidae) Indonesia berdasarkan gen cytochrome B DNA mitokondria, Jurnal Ilmu-Ilmu Hayati 18 (2018) (99–108). [In Bahasa Indonesia]

[10] D. Astuti, M. Irham, H. Ashari, Variasi nukleotida gen COI pada Burung Sempidan (genus *Lophura*) [(nucleotide variations of COI gene on Gallopheasants Bird (genus *Lophura*)], Jurnal Biologi Indonesia 14(1) (2018) 103–108. [In Bahasa Indonesia]

[11] J. Waugh, DNA barcoding in animal species: Progress, potential and pitfalls, BioEssays 29(2) (2007) 188–197. https://doi.org/10.1002/bies.20529.

[12] X.J. Shen, S. Ito, M. Mizutani, Y. Yamamoto, Phylogenetic analysis in chicken breeds inferred from complete cytochrome b gene information, Biochemical Genetics 40(3–4) (2002) 129–141. https://doi.org/10.1023/A:1015165506752.

[13] E. Furlan, J. Stoklosa, J. Griffiths, N. Gust, R. Ellis, R.M. Huggins, A.R. Weeks, Small population size and extremely low levels of genetic diversity in island populations of the platypus, *Ornithorhynchus anatinus*, Ecology and Evolution 2(4) (2012) 844–857. https://doi.org/10.1002/ece3.195

[14] Y. Yuliani, A. Yuniaty, A.H. Susanto, Variasi sekuens DNA yang diamplifikasi menggunakan primer atpB-rbcL pada beberapa kultivar kacang tanah, Scripta Biological 4(1) (2017) 11. https://doi.org/10.20884/1.sb.2017.4.1.377. [In Bahasa Indonesia]

[15] R. Nielsen, Z. Yang, Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene, Jurnal Biologi 936 (1994) (1998) 929–936.