Phylogeography of the Maleo Senkawor (*Macrocephalon maleo* Sal. Muller 1846) Based on Cytochrome B Gene in Sulawesi And Their Sex Determination using Molecular Sexing

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ABSTRAK

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Burung Maleo Senkawor (*Macrocephalon maleo*) mYudaerupakan hewan endemik dan terdistribusi luas di Sulawesi. Burung Maleo Senkawor adalah monomofik sehingga sulit dibedakan antara individu jantan dan betina. Tujuan penelitian ini adalah mengungkap hubungan kekerabatan *M. maleo* di berbagai wilayah geografis di Sulawesi melalui penggunaan penanda gen *Cytochrome-b* (Cyt-b), mengetahui rasio jenis kelamin, dan menguji keandalan primer 2550F/2718R. 15 sampel (bulu dan cangkang telur) berhasil dianalisis dari koleksi di Sulawesi Utara, Sulawesi Tenggara, dan Sulawesi Tengah. Isolasi DNA total dilakukan dengan menggunakan Dneasy® Blood and Tissue kit (Cat. No. 69504) mengikuti protokol Qiagen yang telah dimodifikasi. Amplifikasi PCR (35 siklus) menggunakan primer *forward* MMCytb_F (5'- GAAAATCCCACCCCCTACTA-3') dan primer *reverse* MMCytb_R (5' GTTGGCTACGAGGAGTCAGA-3') serta primer untuk sexing 2550F/2718R. Analisis dilakukan pada sekuens parsial gen Cyt-b mtDNA *M. maleo* sepanjang 903 bp beserta sekuens asam aminonya (301 AA) dan sekuen gen *Chromo Helicase DNA-Binding* (CHD). Jarak genetik model *Kimura -2 Parameter* dan *p-distance*, pada interpopulasi *M. maleo* di Sulawesi (populasi Sulawesi Tenggara dipisahkan dari populasi Sulawesi Tengah dan Sulawesi Utara) dengan nilai 0,002 (0,2%) – 0,003 (0,3%). Tiap-tiap populasi telah terbentuk haplotipe yang berbeda. Primer 2550F/2718R mampu mengamplifikasi gen CHD dan berhasil mengidentifikasi jenis kelaminnya. Individu jantan lebih dominan dibandingkan individu betina dengan rasio jenis kelamin 6,5:1

Kata Kunci: *Macrocephalon maleo*, Phylogeography, Gen Cyt-b, Sex identifikasi, Sulawesi

ABSTRACT

Samad A, Solihin DD, Sumantri C, Purwantara B. 2023.Phylogeography of the Maleo Senkawor (*Macrocephalon maleo* Sal. Muller 1846) based on Cytochrome B Gene in Sulawesi and their sex determination using molecular sexing. JITV 28(1):34-44. DOI: http://dx doi.org/10.14334/jitv/v28i1. 3107.

The Maleo Senkawor bird (*Macrocephalon maleo*) is endemic and widely distributed in Sulawesi. Maleo Senkawor bird is monomorphic so it is difficult to distinguish between male and female individuals. This study aimed to determine the kinship relationship among *M. maleo* in various geographic areas in Sulawesi through the use of the gene marker *Cytochrome-b* (Cyt-b), determine the sex ratio, and examine the reliability of the 2550F/2718R primer. Fifteen (feather and eggshell) samples from North Sulawesi, Southeast Sulawesi, and Central Sulawesi were collected. Total DNA isolation was performed using the *Dneasy® Blood and Tissue kit* (Cat. No. 69504) following a Qiagen protocol with modification. PCR amplification (35 cycles) used a forward primer MMCytb_F (5'- GAAAATCCCACCCCCTACTA-3'), a reverse primer MMCytb_R (5'- GTTGGCTACGAGGAGTCAGA-3') and a primer for sexing used 2550F/2718R. Analysis was performed on the length of 903 bp Cyt-b mtDNA gene sequences of *M. maleo* along with their amino acid sequences (301 AA) and *Chromo Helicase DNA-Binding* (CHD) gene sequences. Based on the genetic distance of the *Kimura 2-Parameter model* and *p-distance*, the interpopulation of *M. maleo* in Sulawesi (the population of Southeast Sulawesi was separated from the population of Central Sulawesi and North Sulawesi) was 0.002 (0.2%) – 0.003 (0.3%). Each population has formed a different haplotype. Primer 2550F/2718R was able to amplify the CHD gene and could distinguish the sex identification. Male individuals are more dominant than female individuals with a sex ratio of 6.5:1.

Key Words: Macrocephalon maleo, Phylogeography, Cyt-b gene, Sex determination, Sulawesi

INTRODUCTION

The Maleo Senkawor bird (*Macrocephalon maleo*) is a species of the Megapodiidae family endemic in Sulawesi island of Indonesia and threatened species by extinction. Many populations were lost due to over-exploitation of their eggs and also lost connectivity between the forest and nesting sites (Arista et al. 2015; Froese & Mustari 2019).

The dispersal of *M. maleo* in Sulawesi formed population groups in nature. Thus, it is suspected that there has been local adaptation and contributed to the emergence of population diversity in each geographical distribution area. There is a need application of genetic marker candidate that able to reveal variations in the interpopulation and conserve requirements for nucleotides characterizing the species. The Cytochrome-b (Cyt-b) gene is one of the genetic markers that have potential to be applied in this study. The *Cytochrome-b* gene is widely used as a marker of differences in geographic distribution and population origin of a species (Çoraman et al. 2013; Ibis et al. 2014; Petrova et al. 2014; Yong et al. 2015; Lapinski et al. 2016; Kato et al. 2016; Anucherngchai et al. 2019; Amiri et al. 2021).

Sources of DNA in birds that are commonly used for genetic analysis can be obtained through non-invasive such as feathers, eggshells, and feces (Begović et al. 2017; Kaunisto et al. 2017; Maia et al. 2017; Drahulian et al. 2018; Rytkönen et al. 2019; Peters et al. 2020; Demarchi et al. 2022). In the present study, DNA was obtained from the feather and eggshell material of the *M. maleo* bird. The use of non-invasive samples was more safe for *M. maleo* compared using a blood sample.

Differentiation and identification of species as well as sex ratios are important steps in conservative interventions (Awad et al. 2015). Therefore, research involving molecular in sex determination of *M. maleo* is important to determine the range of sex ratios in both natural and captive populations. Accurate sex determination is crucial for population development so that it can support the success of conservation programs for endangered species (Purwaningrum et al. 2019). Besides, the activity of egg taking and forest encroachment carried out by humans had a significant effect (87.94%) on the decline in the population of this species (Arista et al. 2015) and also large egg size (length 10.18 cm; width 6.12 cm; weight 211.70 grams) (Widnyanya 2017) attractive for the community to be used as a food.

The Maleo Senkawor is one of the species that have similar phenotypic characteristics between sex. Some basic methods that can be used for sex identification include differences in morphometric characteristics, laparoscopy for sex determination, sex chromosome examination, behavioral observations, and the presence of brooding patches. However, these methods have disadvantages. Sex identification through molecular techniques is more accurate than the other methods. The use of Polymerase Chain Reaction (PCR) method by utilizing specific primers for sex determination has been commonly used in various bird species (Khaerunnisa et al. 2013; Çakmak et al. 2017; Purwaningrum et al. 2019; Osman et al. 2020), but not much has been done on *M. maleo*, especially using materials from feathers and egg shells. One of the genes used in sex determination is Chromo Helicase DNA-Binding (CHD). This CHD gene was amplified by primer 2550F-2781R (Fridolfsson and Ellegren 1999).

The aimed of this study was therefore to reveal the *M. maleo* kinship relationships in different geographic areas in Sulawesi through the use of the gene marker Cytochrome-b (Cyt-b) from Mitochondria DNA with fragment size (903 bp). This Study was also aimed to determine the sex ratio and to examine the reliability of primers in amplification CHD gene with DNA material from feather and eggshell samples of *M. maleo*. The results are expected to support the conservation and development of the *M. maleo* population in Sulawesi island of Indonesia.

MATERIALS AND METHODS

Study area

This research was carried out from July 2019 to January 2021. A total of 15 samples were collected, consisting of 14 feather samples and 1 eggshell sample (hatched). The research location covered 3 regions of North Sulawesi (7 samples), Southeast Sulawesi (5 samples), and Central Sulawesi (3 samples).

Table 1. Primers used in the study

Primers	Sequences (5'– 3')	Genes	Fragment length (bp)
MMCytb_F	GAAAATCCCACCCCCTACTA	Cyt-b	903
MMCytb_R	GTTGGCTACGAGGAGTCAGA		
2550F	GTTACTGATTCGTCTACGAGA	CHD-Z	586
2718R	ATTGAAATGATCCATGCTTG	CHD-W	436

Procedures

Total DNA isolation

Total DNA isolation was carried out from feathers and eggshells using the *Dneasy® Blood and Tissue Kit* (Cat. No. 69504). The procedure used followed on the Spin-Column Protocol procedure from Qiagen (2003) which has been modified by addition of buffer A and buffer B. The sample was then incubated at a temperature of 56 °C (overnight).

Amplification and aequencing of PCR products

The DNA replication process of the Cyt-b gene and CHD gene was carried out through the *Polymerase Chain Reaction* (PCR) technique. A pair of Cyt-b gene primers were designed based on the *M. maleo* genome data in *GenBank* (access code: MW574376.1). The primers were designed through the primer3 program (http://bio-info.ut.ee/primer3-0.4.0/primer3).
Meanwhile, the primers used to amplify the CHD gene are universal primers, namely 2550F/2718R designed by (Fridolfsson and Ellegren 1999). The primers can be seen in the following Table 1.

PCR conditions during the Cyt-b gene amplification process included Pre-denaturation at 94°C for 3 minutes, Denaturation at 94°C for 45 seconds, annealing at 54,45°C for 45 seconds, extension at 72°C for 1 minute with a total of 35 cycles, and post extension 72°C for 6 minutes. Meanwhile, the PCR conditions during the CHD gene amplification process included pre-denaturation at 94°C for 5 minutes, denaturation at 94°C for 45 seconds, annealing at 51,5°C for 45 seconds, an extension at 72°C for 1.5 minutes with a total of 35 cycles, and post extension at 72°C for 10 minutes. Detection of PCR products between the Cyt-b gene and the CHD gene was carried out through electrophoresis on 1.2% agarose gel using TBE-1x buffer. Good DNA bands on target were sequenced through commercial services (*First Base* Malaysia). Identification of the sex of each individual was based on the number of emerged DNA bands. One (1) visible band was identified as a male individual and two (2) bands were identified as female individuals (Vucicevic et al. 2013; Çakmak et al. 2017).

Data analysis

Sequence data of the Cyt-b gene and the CHD gene were aligned using MEGA (*Molecular Evolutionary Genetics Analysis*) software version 7.0 (Kumar et al. 2016). Furthermore, the primer was used to correct the nucleotide sequence to obtain a good sequence. Subsequent sequences were BLAST-n at the National Center for Biotechnology Information (NCBI) site. Reconstruction of the phylogenetic tree was carried out using the *Neighbor-Joining* (NJ) and UPGMA Unweighted Pair Group Method with Arithmetic Average (UPGMA) Kimura 2 – parameter model and p-distance at 1000 bootstraps repetitions. Observation of haplotype diversity used the DNAsp program version 6 (Rozas et al. 2017) and construction of phylogenetic networks was carried out using the Median-Joining method (Bandelt et al. 1999) using the Network program version 10.2 (Fluxus Technology Ltd c2021). Furthermore, 2 sequences from GenBank as an ingroup, namely M. maleo (accession number: AM236881.1 and MW574376.1), and 1 sequence from GenBank as an outgroup, namely Alectura lathami (accession number: AY346091.1).

RESULTS AND DISCUSSION

Result

DNA purity

Quantification of the extracted genomic DNA using the Dneasy® Blood and Tissue Kit (Cat. No. 69504) was carried out to obtain the concentration and purity values of DNA before PCR was performed. The average value of the concentration and purity of DNA from samples of feathers and eggshells of *M. maleo* can be seen in Table 2.

The quantification results in Table 2 showed that the average level of DNA purity is good (1.8-1.9). DNA can be said to be good if the value of the A260/A280 ratio is between 1.8-2.0 (Işçi et al. 2014; Yalçınkaya et al. 2017).

BLASTn result analysis

Sequence data were obtained after being BLASTn on the NCBI site to determine the similarity value to the The search results can be seen in (Tables 3 and Table 4). Table 3 showed that the samples analyzed are Maleo Senkawor birds (*M. maleo*) with a closeness of more than 99%.

Data in Table 4 and Figure 1 showed that seven (7) individuals in the population from North Sulawesi were identified as male. The population from Southeast Sulawesi identified 1 female and four (4) males. Meanwhile, in the population of Central Sulawesi, one (1) female and two (2) male individuals were identified. These results indicated that the ratio of male and female individuals in the analyzed sample is 6,5:1.

Sex determination of *M. maleo* showed that the sequence sizes of the CHDZ and CHDW genes were 586 bp and 436 bp, respectively (Figure 1). In Figure 1, it can be seen that the female individual with 2 bands (CHDZ/CHDW) in the samples from Southeast Sulawesi (MKT1) and Central Sulawesi (MS3) had 586 bp (CHDZ) and 436 bp (CHDW), while the male individual (CHDZ/CHDZ) has only a single band (586 bp).

Table 2. Average concentration and purity of DNA

No	Source of DNA	*N*	concentration (µg/mL)	Purity (A_{260}/A_{280})
1	Feather	14	71.27	1.956
2	Eggshell	1	21.07	1,840

Table 3. Species identification based on BLASTn results in NCBI

Locations	Query cover (%)	E-value	Similarity %	Species	Accession number
North Sulawesi	100	0.0	99.67	*M. maleo*	MW574376.1
	100	0.0	99.56		AM236881.1
Southeast Sulawesi	100	0.0	100	*M. maleo*	MW574376.1
	100	0.0	99.89		AM236881.1
Central Sulawesi	100	0.0	99.78	*M. maleo*	MW574376.1
	100	0.0	99.67		AM236881.1

Table 4. Sex determination based on BLASTn results in NCBI

Species	Sample code	Gene	Fragment length (bp)	Accession number GenBank	Similarity (%)
M. maleo (North Sulawesi-1)	MT1	CHD1-Z	586	MT074328.1	98.35
M. maleo (North Sulawesi-2)	MT2	CHD1-Z	586	MT074328.1	98.35
M. maleo (North Sulawesi-3)	MT3	CHD1-Z	586	MT074328.1	98.35
M. maleo (North Sulawesi-4)	MT4	CHD1-Z	586	MT074328.1	98.35
M. maleo (North Sulawesi-5)	MT5	CHD1-Z	586	MT074328.1	98.35
M. maleo (North Sulawesi-6)	MT6	CHD1-Z	586	MT074328.1	98.35
M. maleo (North Sulawesi-7)	MT7	CHD1-Z	586	MT074328.1	98.35
M. maleo (Southeast Sulawesi-1)	MKT1	CHD1-W	436	MT074330.1	94.10
M. maleo (Southeast Sulawesi-2)	MBHM1	CHD1-Z	586	MT074328.1	95.08
M. maleo (Southeast Sulawesi-3)	MTB1	CHD1-Z	586	MT074328.1	95.23
M. maleo (Southeast Sulawesi-4)	MTB2	CHD1-Z	586	MT074328.1	95.23
M. maleo (Southeast Sulawesi-5)	MTB3	CHD1-Z	586	MT074328.1	95.23
M. maleo (Central Sulawesi-1)	MS1	CHD1-Z	586	MT074328.1	97.80
M. maleo (Central Sulawesi-2)	MS2	CHD1-Z	586	MT074328.1	97.80
M. maleo (Central Sulawesi-2)	MS3	CHD1-W	436	MT074330.1	94.64

Nucleotide sequence polymorphism

The results of multiple alignments of the partial Cyt-b gene (903 bp) Maleo Senkawor (*M. maleo*) btained 898 bp (99.4%) conservative sites, 5 bp (0.6%), parsimony sites 4 bp (0.5%) and 1 bp singleton (0.1%). In this study, 5 polymorphic nucleotide sites were found in the population from Sulawesi and 3 of them indicated specific locations at sites of 678 (Central Sulawesi), 890, and 891 (North Sulawesi) (Table 5).

Haplotype

Based on the data in Tables 5 and Table 4 haplotypes consisted of 3 interpopulation haplotypes and 1 haplotype based on sequences from *GenBank*.

The results of the analysis of Cyt-b gene sequences from 15 samples and 2 comparison sequences from *Genbank* (AM236881.1 and MW574376.1) found 4 haplotypes consisting of 3 interpopulation haplotypes and 1 haplotype in the *GenBank* standard sequence (Figure 2). Haplotype-1 is the sequence from *GenBank* (AM236881.1). Haplotype-2 is the population of Southeast Sulawesi and sequences from GenBank (MW574376.1), Haplotype-3 in the population of North Sulawesi, and haplotype-4 from the population of Central Sulawesi. The value of nucleotide diversity (Pi) in the intrapopulation of *M. maleo* was 0.00000 (0%) and the haplotype diversity (Hd) was 0.00000 (0%). Meanwhile, the interpopulation (3 geographic locations) nucleotide diversity (Pi) was 0.00218 (0.218%) and haplotype diversity (Hd) was 0.71324 (71.324%).

Amino acid sequence polymorphism

The translation results of 903 bp of nucleotide sequences of the partial Cyt-b gene of *M. maleo* obtained 301 amino acid (AA) sites. The results of this analysis provided information that there are 298 conserved AA sites and 2 varied AA encoding sites. The various AA sites are at sites 297 and 300 which are non-synonymous AA (coding nucleotides changed, amino acids changed). Changes occured at 2 sites (297 and 300) are transitional substitutions that occured in pyrimidine bases (Thymine to Cytosine or vice versa) and also in purine bases (Guanine to Adenine or vice versa). Substitution transition occurs at the triplet of codon 2 (890th site of T>C) and the triplet of codon 1 (898th site of G>A). AA substitution occurs at site 297 (from L>P or Leucine to Proline) AA substitution at this site occurs only in populations from North Sulawesi. Meanwhile, the 300th site (A>T or Alanine to Threonine) occurred in populations from North Sulawesi and Central Sulawesi (Table 6).

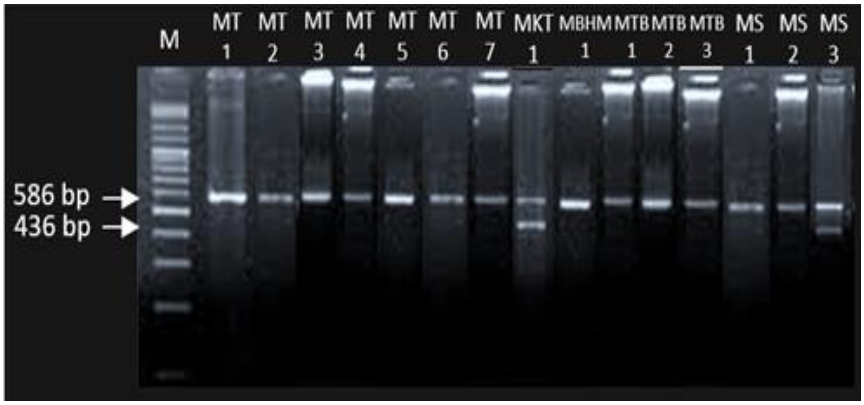

Figure 1. Differences in the size of the *Chromo Helicase DNA-binding* (CHD) gene in *M. maleo.* (M: DNA Ladder 100 bp); MT= Maleo Tambun (North Sulawesi); MKT= Maleo Kolaka Timur (Southeast Sulawesi); MBHM= Maleo Blok Hutan Mempaho (Southeast Sulawesi); MTB= Maleo Tanjung Batikolo (Southeast Sulawesi); MS= Maleo Saluki (Center Sulawesi)

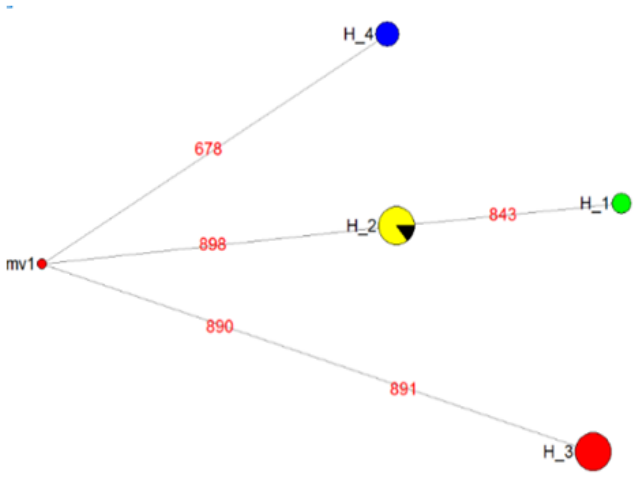

Figure 2. Median-joining network of *M. maleo* interpopulation haplotypes based on Cyt-b gene. where) is H-1 (haplotype-1) *M. maleo* from GenBank (AM236881.1);) is H-2 (haplotype-2) *M. maleo* from Southeast Sulawesi and sequences from GenBank (MW574376.1);) is H-3 (haplotipe-3) *M. maleo* from North Sulawesi;) is H-4 (haplotipe-4) *M. maleo* from Central Sulawesi

Table 5. Nucleotide sequence polymorphisms (SNPs) of the *M. maleo* interpopulation based on the Cyt-b gene

Species	Accession number and sample number	Nucleotide site				
		678 [771]	**843** [936]	**890** [983]	**891** [984]	**898** [991]
M. maleo	AM236881.1	T	A	T	C	G
M. maleo	MW574376.1	T	G	T	C	G
M. maleo (North Sulawesi-1)	MT-1	.	.	C	T	A
M. maleo (North Sulawesi-2)	MT-2	.	.	C	T	A
M. maleo (North Sulawesi-3)	MT-3	.	.	C	T	A
M. maleo (North Sulawesi-4)	MT-4	.	.	C	T	A
M. maleo (North Sulawesi-5)	MT-5	.	.	C	T	A
M. maleo (North Sulawesi-6)	MT-6	.	.	C	T	A
M. maleo (North Sulawesi-7)	MT-7	.	.	C	T	A
M. maleo (Southeast Sulawesi-1)	MKT-1	.	.	.	.	.
M. maleo (Southeast Sulawesi-2)	MBHM-1	.	.	.	.	.
M. maleo (Southeast Sulawesi-3)	MTB-1	.	.	.	.	.
M. maleo (Southeast Sulawesi-4)	MTB-2	.	.	.	.	.
M. maleo (Southeast Sulawesi-5)	MTB-3	.	.	.	.	.
M. maleo (Central Sulawesi-1)	MS-1	C	.	.	.	A
M. maleo (Central Sulawesi-2)	MS-2	C	.	.	.	A
M. maleo (Central Sulawesi-3)	MS-3	C	.	.	.	A

Site-specific nucleotides are marked in black boxes; bold numbers represent nucleotide sequences based on the partial sequence of the Cyt-b gene; the number in brackets is the nucleotide sequence based on the complete genome sequence of the Cyt-b gene.

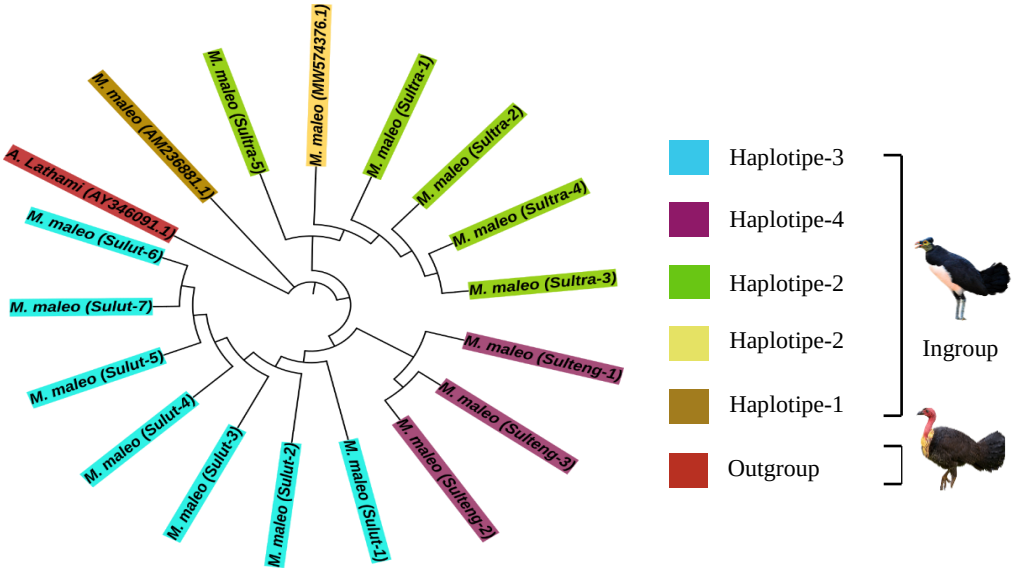

Figure 3. The relationship between populations of *Macrocephalon maleo* based on the Neighbor-Joining (NJ) method, bootstrap 1000 repetitions with the *Kimura 2– parameter* model on the nucleotide sequence of the Cyt-b gene

Table 6. Codon coding sites and amino acids that changed in the Cyt-b gene of *M. maleo*

Population and accession number	Codon site	
	297 (890,891)	300 (898)
AM236881.1	CTC	GCC
MW574376.1	. . .	. . .
North Sulawesi Utara	.CT	A . .
Southeast Sulawesi	. . .	. . .
Central Sulawesi	. . .	A . .
Population and accession number	**amino acid site**	
	297	300
AM236881.1	L	A
MW574376.1	.	.
North Sulawesi	**P**	**T**
Southeast Sulawesi	.	.
Central Sulawesi	.	**T**

The dot symbol (.) is an identical sequence to *M. maleo* from *Genbank* (AM236881.1 and MW574376.1); the bold and underlined numbers = non-synonymous change; the number in parentheses ( )= sequence of nucleotide sites of the Cyt-b *M. maleo* gene

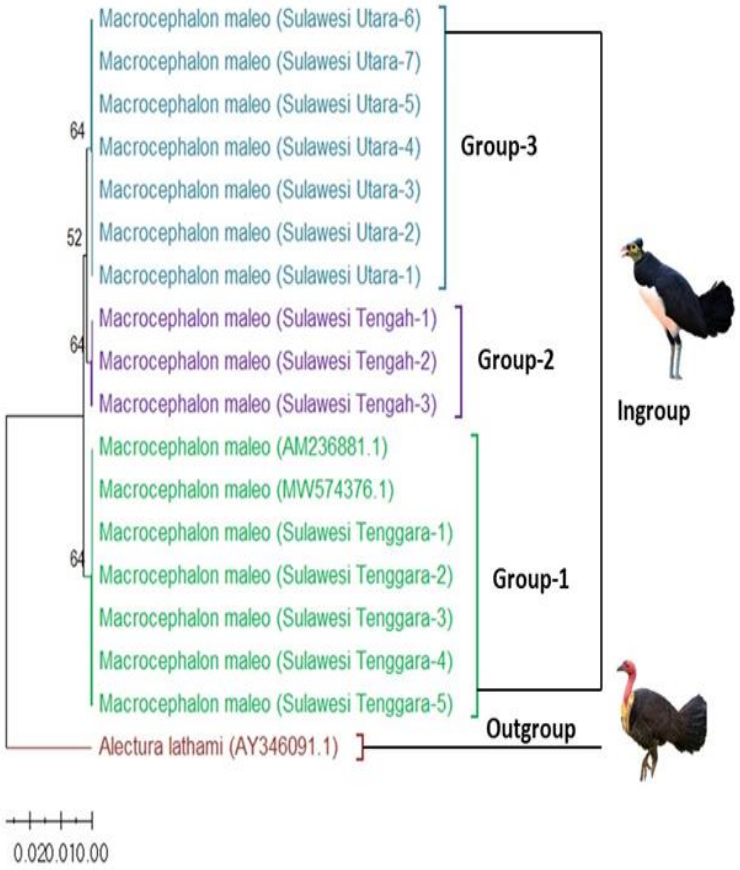

Figure 4. The population between relations of *Macrocephalon maleo* based on the UPGMA (*Unwight Pair Group Method with Arithmetic Average*) method, bootstrap 1000 repetition with the *p-distance* model in the amino acid (AA) sequence

Genetic distance

The genetic distance between species used the Cyt-b *M. maleo* gene in 3 study populations of North Sulawesi, Southeast Sulawesi, and Central Sulawesi with values between 0.002 (0.2%) and 0.003 (0.3%). The genetic distance of the 3 populations when compared with the distance from the *M. maleo* sequence in *GenBank* (AM236881 and MW574376.1) is 0.000 (0.0%) – 0.004 (0.4%). Meanwhile, the genetic distance of *M. maleo* compared to *A. lathami* species (outgroup) was 0.144 (14.4%) – 0.146 (14.6%). The genetic distance based on AA in the 3 study population areas has a value of 0.003 (0.3%)-0.007 (0.7%). The genetic distance in the 3 study populations with comparison sequences from *GenBank* (AM236881 and MW574376.1) was 0.000 (0.0%) – 0.007 (0.3%). Meanwhile, the genetic distance between *M. maleo* and *A. lathami* species (outgroup) was 0.051 (5.1%) - 0.058 (5.8%).

Phylogenetic tree construction

The phylogenetic tree topology of *M. maleo* interpopulation in Sulawesi and 2 reference sequences from GenBank (AM236881.1 and MW574376.1) based on nucleotide sequences of the Cyt-b gene are still in the same cluster (1 cluster). Even though they are in the same cluster, it appears that the populations of *M. maleo* from North Sulawesi and Central Sulawesi tend to have a closer kinship relationship (Figure 3). The phylogenetic tree topology based on AA also shows the same pattern as the nucleotide sequences of the Cyt-b gene and it is known that populations from North Sulawesi and Central Sulawesi tend to have a closer kinship than populations from Southeast Sulawesi (Figure 4)

Discussion

Variations in nucleotide bases in the intrapopulation of *M. maleo* indicated that there is no substitution. Meanwhile, the interpopulation occurs based on a transitional substitution. The results of the analysis showed that transition substitution occured 4 times and no transversion substitution occurs. In *Luscinia calliope*, the dominant transition substitution is based on the Cyt-b gene (Spiridonova et al. 2013). The occurrence of nucleotide base substitution in the interpopulation resulted in the percentage of *M. maleo* genetic distance between North, Southeast, and Central Sulawesi (0.2% - 0.3%.). The low value of the genetic distance of *M. maleo* in the interpopulation indicates that there are no significant genetic differences between the populations of North, Southeast, and Central Sulawesi. This result is presumably because genetic mixing between sub-populations (gene flow) continues to occur so that the differences between different geographic distributions are not significant (the geographic distribution barrier is relatively small). However, geographical variations have occurred between the origin of the geographic distribution of North Sulawesi, Central Sulawesi, and Southeast Sulawesi.

The value of nucleotide diversity and haplotype diversity based on intrapopulation obtained the same value, namely 0.00000 (0%), the low value is suspected whether the population size in each habitat is low or indeed the genetic diversity is low in nature. However, these results provided information that there is a negative impact that could threaten the survival of the *M. maleo* population due to the low intrapopulation nucleotide and haplotype diversity. Low genetic diversity may appear in small populations through inbreeding, thereby triggering negative traits, including decreased fitness, adaptation to environmental changes, reproductive problems, disease susceptibility, and other similar conditions that can reduce population persistence (Pekkala et al. 2014; Fünfstück and Vigilant 2015; Hedrick and Garcia-Dorado 2016; Harrisson et al. 2019).

Based on the pattern formed in the haplotype network, the Southeast Sulawesi sub-population and the sequence from GenBank (MW574376.1) are combined into the same haplotype, namely Haplotype-2. This condition is because this sub-population is still composed of common nucleotides found in all populations. Meanwhile, the sub-populations of Central Sulawesi, North Sulawesi, and sequences from *Genban*k (AM236881.1) appeared to form separate sub-populations.

The existence of *M. maleo* in Sulawesi as a species in the Family Megapodiidae that has evolved, it might be thought to have originated from an ancestor in Australia. Megapodiidae existed in Australia in the late Oligocene and there is general agreement that Megapodiidae were isolated in Australia-Papua for a long time (Harris et al. 2014). In addition, *M. maleo* reaching the island of Sulawesi is inseparable from the process of forming this island. It is suspected that Sulawesi originated from Mainland Asia, Mainland Australia, and lands arising from the ocean floor during the Miocene to Pleistocene epochs (Hall 2001; Shekelle and Leksono 2019). *M. maleo* colonization on Sulawesi Island is directly related to the geological formation and source of its initial population and evolution of development.

Based on the results of sex identification, it is known that from the 15 samples analyzed, 13 individuals were male and 2 individuals were female. The dominance of male individuals over female individuals occurred in 3 study population areas. The sex ratio plays an important role and influences sex roles and the reproductive system (Szekely et al. 2014). An unbalanced sex ratio in a small population can result in a decline of population that leads to the extinction of the species (Kus et al. 2017) and this

certainly harms *M. maleo* as an endangered species. Sex determination using primers 2550F and 2718R (Fridolfsson and Ellegren 1999) was able to distinguish the sex of the *M. maleo* bird species with DNA sources from feathers and eggshells. The size of the CHDW and CHDZ fragments through the primary determination of 2550F/2718R varied, namely 440 bp -705 bp (Çakmak et al. 2017), 600 bp-100 bp (Ravindran et al. 2019). The results of this study were not significantly different from those that had been done previously on CHD-Z *M. maleo* (Yuda and Saputra 2021).

Habitat damage, especially in coastal areas in Sulawesi, which is one of the spawning habitats of *M. maleo* is very massive because it is used as an area for agriculture, aquaculture, and settlements. The decreasing number of nests of *M. maleo* is thought to be strongly influenced by the deterioration of conditions and the loss of the ideal nesting habitat for regeneration. Thus, the population declined and the limited geographical distribution of this animal are becoming increasingly limited and population development is increasingly worrying. Many areas where previously this animal species could be found, are now empty. In addition ~~to~~ habitat destruction, egg hunting ~~is~~ are also a serious threat to the survival of this species, because the egg size is large so it is interesting for human consumption.

CONCLUSION

Based on genetic markers, the Cyt-b gene is large (79% of total length) indicating that populations from North Sulawesi and Central Sulawesi have a closer kinship with a genetic distance marker of 0.2%. Meanwhile, the population from Southeast Sulawesi is seen to be separated from other populations with a genetic distance of 0.3%. Although they are still in the same cluster (populations of North Sulawesi and Central Sulawesi), each population has its haplotype, namely haplotype-3 in populations from North Sulawesi and haplotype-4 in populations from Central Sulawesi. The Southeast Sulawesi population belongs to the sequence from GenBank (MW574376.1) has its haplotype, namely haplotype-2, and is in a separated cluster. Primers 2550F and 2718R were able to amplify the CHD gene in *M. Maleo* with fragment sizes for CHDZ and CHD W~~,~~ were 546 bp and 436 bp, respectively. Meanwhile, sex identification showed that the male sex was dominant over the female sex with a ratio of 6,5:1

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REFERENCES

Amiri N, Vaissi S, Aghamir F, Saberi-Pirooz R, Rödder D, Ebrahimi E, Ahmadzadeh F. 2021. Tracking climate change in the spatial distribution pattern and the phylogeographic structure of Hyrcanian wood frog, Rana pseudodalmatina (Anura: Ranidae). J Zool Syst Evol Res. 59:1604–1619. DOI:10.1111/jzs.12503.

Anucherngchai S, Chontananarth T, Tejangkura T, Chai JY. 2019. The study of Cytochrome B (CYTB): species-specific detection and phylogenetic relationship of Echinostoma revolutum, (Froelich, 1802). J Parasit Dis. 43:66–74. DOI:10.1007/s12639-018-1057-0.

Arista K, Wahid A, Ihsan M. 2015. Faktor penyebab penurunan populasi Maleo Senkawor di Desa Sausu Piore, Kabupaten Parigi Moutong, Sulawesi Tengah. War Rimba. 3:1–8.

Awad A, Khalil, Abd-Elhakim. 2015. Molecular phylogeny of some avian species using Cytochrome b gene sequence analysis.

Bandelt H-J, Forster P, Röhl A. 1999. Median-Joining Networks for Inferring Intraspecific Phylogenies. Mol Biol Evol. 16:37–48.

Begović L, Mihić I, Pospihalj T, Mikuška T, Mlinarić S, Mikuška A. 2017. Evaluation of methods for molecular sex-typing of three heron species from different DNA sources. Turkish J Zool. 41:593–598. DOI:10.3906/zoo-1602-47.

Çakmak E, Akın Pekşen Ç, Bilgin CC. 2017. Comparison of three different primer sets for sexing birds. J Vet Diagnostic Investig. 29:59–63. DOI:10.1177/1040638716675197.

Çoraman E, Furman A, Karataş A, Bilgin R. 2013. Phylogeographic analysis of Anatolian bats highlights the importance of the region for preserving the Chiropteran mitochondrial genetic diversity in the Western Palaearctic. Conserv Genet. 14:1205–1216. DOI:10.1007/s10592-013-0509-4.

Demarchi B, Stiller J, Grealy A, Mackie M, Deng Y, Gilbert T, Clarke J, Legendre LJ, Boano R, Sicheritz-Ponten T, Magee J, Zhang G, Bunce M, Collins MJ, Miller G. 2022. Ancient proteins resolve controversy over the identity of Genyornis eggshell. PNAS. e2109326119. DOI:10.1073/pnas.2109326119 j.

Drahulian M, Chaplygina A, Savynska N, Kostenko S, Ostrovskyi P, Gusar K. 2018. The physiological and genetic differences between flycatchers (Ficedula albicollis vs. Ficedula hypoleuca). Folia Oecologica. 45:111–119.DOI:10.2478/foecol-2018-0012

Fridolfsson A-K, Ellegren H. 1999. Short Communications. J AVIAN Biol. 30:116–121. DOI:10.1163/003925995X00233.

Froese GZL, Mustari AH. 2019. Assessments of Maleo Macrocephalon maleo nesting grounds in South-east Sulawesi reveal severely threatened populations. Bird Conserv Int. 29:497–502. DOI:10.1017/S0959270918000333

Fünfstück T, Vigilant L. 2015. The geographic distribution of genetic diversity within gorillas. Am J Primatol. 77:974–985. DOI:10.1002/ajp.22427.

Hall R. 2001. Cenozoic reconstructions of SE Asia and the SW Pacific: Changing patterns of land and sea. Faunal Flor Migr Evol SE Asia-Australasia.(Figure 1):35–56.

Harris RB, Birks SM, Leaché AD. 2014. Incubator birds: Biogeographical origins and evolution of underground nesting in megapodes (Galliformes: Megapodiidae). J Biogeogr. 41:2045–2056. DOI:10.1111/jbi.12357.

Harrisson KA, Magrath MJL, Yen JDL, Pavlova A, Murray N, Quin B, Menkhorst P, Miller KA, Cartwright K, Sunnucks P. 2019. Lifetime Fitness Costs of Inbreeding and Being Inbred in a Critically Endangered Bird. Curr Biol 29 (16): 2711-2717. DOI:10.1016/j.cub.2019.06.064.

Hedrick PW, Garcia-Dorado A. 2016. Understanding Inbreeding Depression, Purging, and Genetic Rescue. Trends Ecol Evol. 31:940–952. DOI:10.1016/J.TREE .2016.09.005.

Ibis O, Tez C, Özcan S. 2014. Phylogenetic Status of the Turkish Red Fox (*Vulpes vulpes*), based on Partial Sequences of the Mitochondrial Cytochrome-b Gene. *Vertebrate Zoology.* 64: 273 – 284.

Işçi B, Yildirim HK, Altindisli A. 2014. Evaluation of methods for DNA extraction from must and wine. *Journal of the Institute of Brewing. 120*(3): 238-243. DOI:10.1002/jib.129.

Kaunisto KM, Roslin T, Sääksjärvi IE, Vesterinen EJ. 2017. Pellets of proof: First glimpse of the dietary composition of adult odonates as revealed by metabarcoding of feces. Ecol Evol. 7:8588–8598. DOI:10.1002/ece3.3404.

Kato H, Gomez EA, Martini-Robles L, Muzzio J, Veles L, Calpovina M, Romero-Alvares D, Mimori T, Uezato H, Hashiguchi Y. 2016. Geographic distribution of *Leishamina* species in Ecuador based on the Cytochrome B gene sequence analisys. PLoS Negl Trop Dis 10: e0004844. DOI:10.1371/journal.pntd.0004844.

Khaerunnisa I, Sari E, Ulfah M, Jakaria, Sumantri C. 2013. Avian sex determination based on chromo helicase DNA-binding (CHD) genes using polymerase chain reaction (PCR). Media Peternak. 36:85–90. DOI:10.5398/medpet.2013.36.2.85.

Kumar S, Stecher G, Tamura K, Dudley J. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 33:1870–1874. DOI:10.1093/molbev/msw054.

Kus BE, Howell SL, Wood DA. 2017. Female-biased sex ratio, polygyny, and persistence in the endangered Southwestern Willow Flycatcher (*Empidonax traillii extimus*). 119:17–25. DOI:10.1650/CONDOR-16-119.1.

Lapinski AG, Pavlenko MV, Solovenchuk LL, Gorbachev V V. 2016. Some limitations in the use of the mitochondrial DNA cytb gene as a molecular marker for phylogenetic and population-genetic studies by the example of the Apodemus genus. Russ J Genet Appl Res. 6:84–90. DOI:10.1134/S2079059716010093.

Maia TA, Vilaça ST, da Silva LR, Santos FR, Dantas GPDM. 2017. DNA sampling from eggshells and microsatellite genotyping in rare tropical birds: Case study on Brazilian Merganser. Genetics and Molecular Biology. 40: 808-812. DOI:10.1590/1678-4685-GMB-2016-0297.

Osman MA, Sugnaseelan S, Jothi |, Panandam M, Putra Malaysia U, Correspondence M, Izza N, Ghani A. 2020. Molecular sex identification of Malaysian White-Nest Swiftlet (Aerodramus fuciphagus Thunberg, 1812) [Internet]. https://doi.org/10.1002/ece3.6699

Pekkala N, Knott KE, Kotiaho JS, Nissinen K, Puurtinen M. 2014. The effect of inbreeding rate on fitness, inbreeding depression and heterosis over a range of inbreeding coefficients. *7*(9): 1107-1119. DOI:10.1111/eva.12145.

Peters C, Nelson · Howard, Rusk B, Muir A. 2020. A novel method to optimise the utility of underused moulted plumulaceous feather samples for genetic analysis in bird conservation. 12:457–467. DOI:10.1007/s12686-019-01117-8.

Petrova TV, Zakharov ES, Samiya R, Abramson NI. 2014. Phylogeography of the narrow-headed vole *Lasiopodomys* (*Stenocranius*) *gregalis* (Cricetidae, Rodentia) inferred from mitochondrial cytochrome b sequences: an echo of Pleistocene prosperity. J Zoolog Syst Evol Res. *53*(2): 97-108. DOI: 10.1111/jzs.12082.

Purwaningrum M, Nugroho HA, Asvan M, Karyanti K, Alviyanto B, Kusuma R, Haryanto A. 2019. Research Centre for Biology, Indonesian Institute of Sciences. Gembira Loka Zoo Bot Gard. 2. DOI:10.14202/ vetworld.2019.1506-1513.

Ravindran S, Woo WK, Saufi S, Amni WN, Hamid NH, Abidin CMRZ, Ishak I, Azzam G, Salim H. 2019. Molecular sexing of southeast asian barn owl, tyto alba javanica, using blood and feather. Trop Life Sci Res. 30:13–23. DOI:10.21315/tlsr2019.30.2.2.

Rozas J, Ferrer-Mata A, Carlos anchez-DelBarrio JS, Guirao-Rico S, Librado P, an Ramos-Onsins SE, anchez-Gracia AS. 2017. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. DOI:10.1093/mol bev/msx248.

Rytkönen S, Vesterinen EJ, Westerduin C, Leviäkangas T, Vatka E, Mutanen M, Välimäki P, Hukkanen M, Suokas M, Orell M. 2019. From feces to data: A metabarcoding method for analyzing consumed and available prey in a bird-insect food web. Ecol Evol. 9:631–639. DOI:10.1002/ece3.4787.

Shekelle M, Leksono SM. 2019. Strategi konservasi di Pulau Sulawesi dengan menggunakan tarsius sebagai flagship spesies. Biota J Ilm Ilmu-Ilmu Hayati. 9 (1): 1-10. DOI:10.24002/biota.v9i1.2791.

Spiridonova LN, Val'chuk OP, Belov PS, Maslovsky KS. 2013. Intraspecific genetic differentiation of the Siberian Rubythroat (*Luscinia calliope*): Data of sequencing the

mtDNA cytochrome b gene. Russ J Genet. 49:638–644. DOI:10.1134/S1022795413060136.

Szekely T, Weissing FJ, Komdeur J. 2014. Adult sex ratio variation: implications for breeding system evolution. J Evol Biol. 27:1500–1512. DOI:10.1111/jeb.12415.

Vucicevic M, Stevanov-Pavlovic M, Stevanovic J, Bosnjak J, Gajic B, Aleksic N, Stanimirovic Z. 2013. Sex determination in 58 bird species and evaluation of CHD Gene as a universal molecular marker in bird sexing. Zoo Biol. 32:269–276. DOI:10.1002/zoo.21010.

Widnyanya I. 2017. Kajian morfologi dan morfometri telur burung Maleo (*Macrocephalon maleo* SAL. Muller 1846). Agropet. 14(1). ISSN:1693-9158.

Yalçınkaya B, Yumbul E, Mozioğlu E, Akgoz M. 2017. Comparison of DNA extraction methods for meat analysis. Food Chem. 221:1253–1257. DOI:10.1016/J.FOODCHEM.2016.11.032.

Yong H Sen, Eamsobhana P, Song SL, Prasartvit A, Lim PE. 2015. Molecular phylogeography of Angiostrongylus cantonensis (Nematoda: Angiostrongylidae) and genetic relationships with congeners using cytochrome b gene marker. Acta Trop. 148:66–71. DOI:10.1016/J.ACTATROPICA.2015.04.020.

Yuda P, Saputra AW. 2021. Eggshell membrane for DNA sexing of the endangered Maleo (*Macrocephalon maleo*). Version 4 F1000Research. 9:599. DOI:10.12688/f1000research.23712.1.