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DNA barcoding of *Macrocephalon maleo* originating from Sulawesi, Indonesia

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Abstract. *Samad A, Solihin DD, Sumantri C, Purwantara B. 2022. DNA barcoding of* Macrocephalon maleo *originating from Sulawesi, Indonesia. Biodiversitas 23: 4400-4408.* Conservation of Maleo Senkawor (*Macrocephalon maleo* Sal. Muller, 1846) continues to be carried out to sustain this bird species. The genetic information from this study can be used to support future conservation activities. This study aimed to provide information on the genetic characteristics of the endangered Maleo Senkawor based on the partial sequence of the Cytochrome Oxidase 1 (COI) gene in mitochondrial DNA. Samples were collected from Central Sulawesi, Southeast Sulawesi, and North Sulawesi. Total DNA isolation was performed using the Dneasy® Blood and Tissue kit through a modified Qiagen protocol. PCR amplification used a forward primer MMCOI_F (5'-ccgatgattattctcaacca-3') and a reverse primer MMCOI_R (5'-gtagccgaatggttcttttt-3'). Analysis was performed on the partial sequences of the *M. maleo* COI mtDNA gene and its amino acid sequences. The genetic distance between the *M. maleo* population from North Sulawesi, Southeast and Central Sulawesi is 0.001 (0.1%). *Macrocephalon maleo* population of Southeast and Central Sulawesi have 0% genetic distance. Analysis of the COI *M. maleo* gene from 3 population locations showed that they came from the same species. Based on the genetic distance of the Kimura model with 2 parameters in the 3 populations, the *M. maleo* formed the same group (ingroup species) but had a different haplotype. These differences can be used as characterizing markers in each population. Furthermore, the conservation of *M. maleo* from these 3 populations was better treated as a separate group based on the mitochondrial DNA COI gene to avoid the negative impact of interpopulation breeding.

Keywords: Conservation, Cytochrome Oxidase 1 (COI), endemic, genetic, mtDNA

INTRODUCTION

The most significant biodiversity hotspot in the Wallacea area is the island of Sulawesi, which contains endemic fauna whose population abundance is significantly declined (Priston et al. 2012; Stelbrink et al. 2012; Tasirin et al. 2021). The Maleo Senkawor bird (*Macrocephalon maleo* Sal. Muller, 1846) is an iconic endemic bird of Sulawesi Island, Indonesia (Butchart and Baker 2000; Frose and Mustari 2019; Yuni and Yuda 2020; Tasirin et al. 2021). This bird is unique to the Megapodiidae family because the strategy in incubating its eggs has changed completely depending on environmental heat sources (Harris et al. 2014; Frose and Mustari 2019). Previous studies stated that there was a sharp decline in the population of the Maleo Senkawor (Frose and Mustari 2019; BirdLife 2021). Butchart and Baker (2000) explained that Over 70% of populations of Maleo Senkawor were declining, and only half of the active nests were protected. Egg hunting is a major threat to the survival of this species. Habitat destruction (forest degradation, deforestation and habitat fragmentation) cuts connectivity between nesting sites and forests, potentially increasing chick mortality (Indrawan et al. 2012; Tasirin et al. 2021). As a result, Maleo Senkawor is listed on the International Union for Conservation of Nature (IUCN) red list as an endangered species (BirdLife 2021). Maleo Senkawor has a high risk of extinction based on these problems, so conservation efforts to preserve this bird are important, both through in-situ and ex-situ conservation efforts.

Molecular studies have an important role in presenting genetic database information of a species that can be used as a basis or conservation guideline in the future (Pekkala et al. 2014; Hedrick and Garcia-Dorado 2016). Data were obtained through a series of scientific studies, including molecular species identification (barcoding), genetic variation and adaptation to environmental changing responses. Conservation approaches involving molecular parts have been applied to the Andean Condor *(Vultur gryphus*) and the California Condor (*Gymnogyps californianus*) (Padro et al. 2020). The results showed that the Andean condor lost about 17% of genetic variability compared to the California Condor and these species also used the same demographic area.

One of the reliable molecular markers widely used in animal species genetic studies is the Cytochrome Oxidase 1

(COI) gene, especially for bird species (Colihueque et al. 2021). This marker is isolated from mitochondrial DNA (mtDNA). mtDNA is an effective marker to indicate evolutionary rate, haplotype diversity, and species origin (Krebs et al. 2017; Pârâu et al. 2019). This gene is used as an effective DNA barcode to define animal species boundaries and was applied in several bird studies. This gene can be amplified by several sets of primers (Yu et al. 2012; Aliabadian 2013; Deagle et al. 2014; Hill 2016). Identification of bird species in South America, such as in Brazil, Ecuador and French Guiana, showed that COI gene sequences were highly accurate for species-level identification (93%-98%) (Milá et al. 2012; Chaves et al. 2015). Joo and Park (2012) identified 39 samples of bird feces in South Korea and obtained a genetic distance value of 0.5% intraspecies and 9.1% intragenus. In addition, identifying 58 smuggled eggs at Brazilian airports revealed that 57 embryos were parrot species and one embryo was an owl (Goncalves et al. 2015).

Research related to the genetic diversity of *M. maleo* has been carried out by Saputra and Yuda (2020) using the Control Region mtDNA hypervariable of *M. maleo*. The results showed that the nucleotide diversity of *M. maleo* was relatively low, 0.727270 in the coastal area of Tanjung Binerean and 0.848480 in the Tambun Forest habitat (North Sulawesi). On the other hand, research has never been carried out using COI gene markers to determine the species identity and genetic diversity of *M. maleo* in a wider area in Sulawesi. This study aimed to provide genetic characteristics information of the endangered Maleo Senkawor based on the partial sequence of the Cytochrome Oxidase 1 (COI) gene in mitochondrial DNA in 3 regions: North Sulawesi, Southeast Sulawesi and Central Sulawesi.

MATERIALS AND METHODS

Study area

This study was conducted from July 2019 to January 2021. The study locations were in North Sulawesi, Southeast Sulawesi and Central Sulawesi, Indonesia (Figure 1). Geographically, the population of *M. maleo* is distributed in Southeast Sulawesi, Central Sulawesi, and North Sulawesi (BirdLife 2021). The total of samples was 26: North Sulawesi (n: 12), Southeast Sulawesi (n: 10), and Central Sulawesi (n: 4). The 22 feather samples were collected from Maleo Senkawor nesting sites, and four samples were collected from hatched eggshells. The feathers and eggshells were collected from different nesting sites and it can be assumed that the samples came from different individuals.

The sample was collected from bird sanctuaries and wildlife. Material, sample code and sample coordinate can be seen in Table 1.

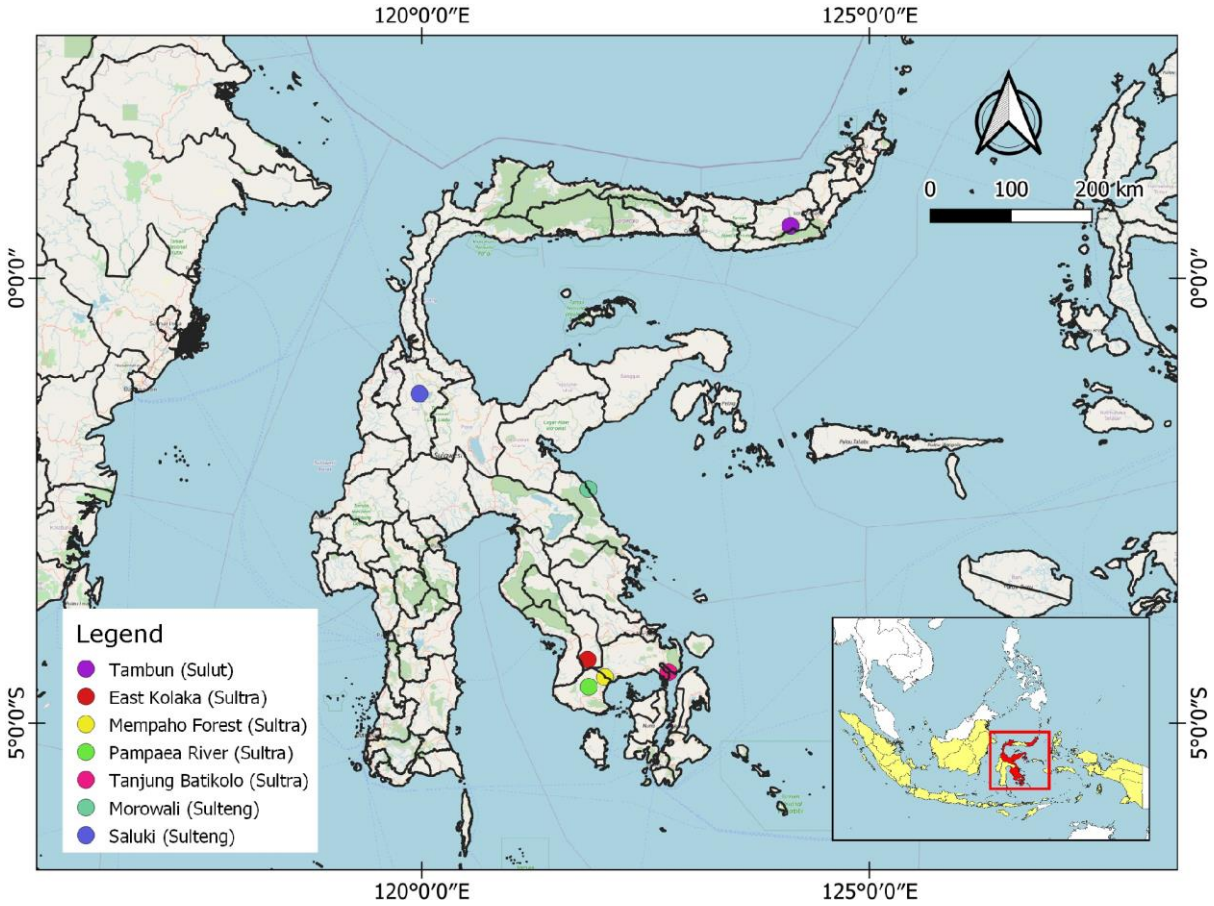


Figure 1. Sampling locations map in North Sulawesi, Southeast Sulawesi, and Central Sulawesi, Indonesia

Table 1. Sample data information

Region	Sample location	Sample source	Material and sample code	*n*	Coordinate
North Sulawesi, Indonesia	Tambun	Bird sanctuary	Feather (MT)	12	00° 35'19.32" N; 124° 07' 06.95" E
Southeast Sulawesi, Indonesia	Tanjung Batikolo Wildlife Reserve	Wildlife	Feather (MTB)	5	04°25'35.33" S; 122°45'22.90" E
	Pampaea River	Wildlife	Eggshell (MSP)	2	04°35'23.00'' S; 121°52'00.00'' E
	Mempaho Forest	Wildlife	Eggshell (MBHM)	1	04°28'51.25" S; 122°02'45.57" E
	East Kolaka	Wildlife	Feather (MKT)	2	04°17'06.00'' S; 121°51'09.00'' E
Center Sulawesi, Indonesia	Saluki	Bird sanctuary	Feather (MS)	3	01°17'54.00'' S; 119°58'32.00'' E
	Morowali	Wildlife	Eggshell (MM)	1	02°22'08.00'' S; 121°51'44.00'' E

Procedures

Total DNA isolation

Total DNA isolation was carried out on feathers and eggshells. Feathers and egg shells were stored separately in zip-lock plastic bags and labeled according to sample location. Furthermore, the samples were stored in silica gel and sent to the laboratory. Samples were stored at -18°C until total DNA extraction was performed.

The total DNA isolation process was carried out using the Dneasy® Blood and Tissue Kit cat number 69504 (50). The isolation procedure was based on the Spin-Column Protocol procedure from Qiagen (2003), which has been modified by adding buffer A and buffer B.

Amplification and sequencing of PCR products

The process of COI gene DNA replication was performed using Polymerase Chain Reaction (PCR) technique. The primers were designed using the primer3 program (http://bio-info.ut.ee/primer3-0.4.0/primer3). These primers are expected to be able to amplify the COI mtDNA gene of *M. maleo*. The results of the primary design for the COI gene were the forward primer MMCOI_F (5'-CCGATGATTATTCTCAACCA-3') and the reverse primer MMCOI_R (5'-GTAGCCGAATGGTTCTTTTT-3'). The length of the amplification product fragment is about 796-bp.

The PCR conditions were Predenaturation at 94°C for 3 minutes, Denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds, extension at 72°C for 1 minute with a total of 35 cycles and post PCR, and post extension 72°C for 6 minutes. Furthermore, the detection of PCR products was carried out through electrophoresis on 1.2% agarose gel using TBE-1x buffer. DNA bands that were clear and on target will be sequenced at First Base Malaysia.

Data analysis

The data from the sequencing process were aligned using MEGA (Molecular Evolutionary Genetics Analysis) software version 7.0 (Kumar et al. 2016). Primer MMCOI_F and primer MMCOI_R were used to correct the nucleotide sequences to obtain clear and on-target sequences. Furthermore, a query was performed on the sequences using the BLAST-n program on the National Center for Biotechnology Information (NCBI) website. Reconstruction of the phylogenetic tree was carried out using the Neighbor-Joining, Kimura 2-parameter model, and p-distance with 1000 bootstrap repetitions. Haplotype diversity was identified using DNAsp program version 510.01 (Rozas 2009) and Network version 5 (Bandelt et al. 1999).

In this analysis, the Australian Brush-turkey (*Alectura lathami*) from GenBank (MN356204.1) was used as the outgroup species. *Macrocephalon maleo* and *A. lathami* are from the same family, Megapodiidae. In addition, *A. lathami* has complete genetic sequence data in GenBank, especially regarding mitochondrial DNA.

RESULTS AND DISCUSSION

BLASTn result analysis

BLASTn analysis results showed that 22 feather and 4 eggshell samples were identified as species of *M. maleo*. The accuracy from sample locations was >99% and E-value <0.0001 (Table 2).

Nucleotide sequence polymorphisms of the COI gene

The multiple alignments of the partial COI gene (796 bp) showed that *M. maleo* had conserved sites 792 bp (99.5%), variable sites 4 bp (0.5%), parsimony sites bp (0.1%) and singleton 3 sites (0.4%). The composition of nucleotide bases was 26.1% thymine base, 33% cytosine base, 24.8% adenine base, and 16.1% guanine base. The composition percentage of A+T and G+C nucleotide bases were 50.9% and 49.1%, respectively.

The genetic analysis result of the Maleo Senkawor interpopulation from Sulawesi had 1 site of Single Nucleotide Polymorphism (SNP), the distinguishing feature of the population from North Sulawesi. On the other hand, the population from North Sulawesi, which was compared to sequences from GenBank (MW574376.1), had 4 SNP sites (sites 5, 13, 28, and 790). Site 28 is a specific site and acts as a differentiator from the other 2 populations (Southeast Sulawesi and Central Sulawesi). Southeast Sulawesi and Central Sulawesi populations which were compared to sequences from GenBank (MW574376.1), had 3 SNP sites (5, 13, and 790) (Table 3).

Haplotype

In this study, 3 haplotypes were found based on the COI gene sequences from 26 samples and 1 comparison sequence from Genbank. Haplotype-1 is *M. maleo* from GenBank (MW574376.1). Haplotype-2 came from the population of North Sulawesi. Haplotype-3 came from the population of Southeast Sulawesi and Central Sulawesi.

There were 3 mutation points in haplotype-1 and haplotype-3, which were sites 5, 13, and 790. Haplotype-3 and haplotype-2 had 1 point mutation, which was site 28 (Figure 2). This analysis shows that few mutations occur between different geographic distributions in Sulawesi. Geographic isolation produces a unique phenotype and causes mutations (Worsham et al. 2017).

Median Joining Network analysis result showed that sequences from GenBank (MW574376.1) formed haplotype-1, populations from North Sulawesi formed haplotype-2, and populations from Southeast Sulawesi and Central Sulawesi formed haplotype-3. The formed pattern in the haplotype network shows that haplotype-3 is dominant. Yuan et al. (2009) explained that the dominant haplotype is the main radiation center of a population. In other words, the *M. maleo* population of South and Southeast Sulawesi is the main radiation center of the *M. maleo* population. High diversity of haplotype showed that *M. maleo* is endemic species (Astuti and Prijono 2018).

Amino acid sequence polymorphism

In this study, there were 263 amino acid (AA) sites translated from 796 bp of partial COI gene nucleotide sequences, 259 conserved AA sites, and 4 polymorphic AA sites (2, 5, 10, and 262). Based on the results of the alignment with the comparison sequence, these results indicate that the mutation that occurs at these sites are non-synonymous mutations (Table 4).

Table 4. The number of conservative and non-synonymous amino acid sites toward the total amino acids translated from the *Macrocephalon maleo* COI gene

Amino acid site	Total
Conservative	259 / 263
Non-synonymous	4 / 263

Table 2. Species identification based on BLASTn results

Location	Query cover (%)	E - value	Identity (%)	Conspecific references	Accession number
North Sulawesi	100	0.0	99.37	*Macrocephalon maleo*	MW574376.1
Southeast Sulawesi	100	0.0	99.62	*Macrocephalon maleo*	MW574376.1
Central Sulawesi	100	0.0	99.62	*Macrocephalon maleo*	MW574376.1

Table 3. Nucleotide sequence polymorphisms of *Macrocephalon maleo* interpopulation based on the COI gene

Species	Accession number and sample number	Nucleotide site			
		5	13	28	790
Macrocephalon maleo (GenBank)	MW574376.1	C	T	C	T
Macrocephalon maleo (North Sulawesi -1)	MT-1	T	A	A	C
Macrocephalon maleo (North Sulawesi -2)	MT-2	T	A	A	C
Macrocephalon maleo (North Sulawesi -3)	MT-3	T	A	A	C
Macrocephalon maleo (North Sulawesi -4)	MT-4	T	A	A	C
Macrocephalon maleo (North Sulawesi -5)	MT-5	T	A	A	C
Macrocephalon maleo (North Sulawesi -6)	MT-6	T	A	A	C
Macrocephalon maleo (North Sulawesi -7)	MT-7	T	A	A	C
Macrocephalon maleo (North Sulawesi -8)	MT-8	T	A	A	C
Macrocephalon maleo (North Sulawesi -9)	MT-9	T	A	A	C
Macrocephalon maleo (North Sulawesi -10)	MT-10	T	A	A	C
Macrocephalon maleo (North Sulawesi -11)	MT-11	T	A	A	C
Macrocephalon maleo (North Sulawesi -12)	MT-12	T	A	A	C
Macrocephalon maleo (Southeast Sulawesi -1)	MTB-1	T	A	.	C
Macrocephalon maleo (Southeast Sulawesi -2)	MTB-2	T	A	.	C
Macrocephalon maleo (Southeast Sulawesi -3)	MTB-3	T	A	.	C
Macrocephalon maleo (Southeast Sulawesi -4)	MTB-4	T	A	.	C
Macrocephalon maleo (Southeast Sulawesi -5)	MTB-5	T	A	.	C
Macrocephalon maleo (Southeast Sulawesi -6)	MSP-1	T	A	.	C
Macrocephalon maleo (Southeast Sulawesi -7)	MSP-2	T	A	.	C
Macrocephalon maleo (Southeast Sulawesi -8)	MBHM-1	T	A	.	C
Macrocephalon maleo (Southeast Sulawesi -9)	MKT-1	T	A	.	C
Macrocephalon maleo (Southeast Sulawesi -10)	MKT-2	T	A	.	C
Macrocephalon maleo (Central Sulawesi -1)	MM-1	T	A	.	C
Macrocephalon maleo (Central Sulawesi -2)	MS-1	T	A	.	C
Macrocephalon maleo (Central Sulawesi -3)	MS-2	T	A	.	C
Macrocephalon maleo (Central Sulawesi -4)	MS-3	T	A	.	C

Note: A: Adenine; C: Cytosine; T: Thymine

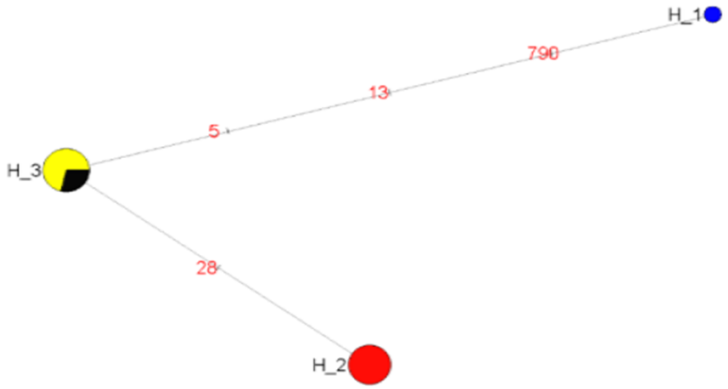


Figure 2. Median of joining network haplotypes of *Macrocephalon maleo* interpopulation based on COI gene. Where: (●): H-1 (haplotypes-1) *Macrocephalon maleo* GenBank (MW574376.1); (●): H-2 (haplotypes -2) North Sulawesi; (●): H-3 (haplotypes -3) Southeast Sulawesi and Central Sulawesi

The polymorphism sites which was found in the COI gene sequences of *M. maleo* were caused by the substitution of nucleotide bases. The results of the analysis on the triplet codon between nucleotide sequence from the *M. maleo* interpopulation and GenBank (MW574376.1) found 4 nucleotide substitutions (2 transition substitutions and 2 transversion substitutions) (Table 5). Transition substitution occurred at site 5 C→T and at site 790 T→C (haplotype-2 and haplotype-3 were identical). Transversion substitution occurred at site 13 T→A (haplotype-2 and haplotype-3 were identical). At site 28, coding sequence mutation only occurred in haplotype-2 C→A (haplotype-1 and haplotype-3 were identical). The nucleotide site that changes causes a mutation in the amino acid composition (non-synonymous mutation). Mutations can occur due to variations in nucleotide bases. According to Matter et al. (2009), Small variations affect the similarity of a species and also affect the arrangement of amino acids that code for proteins.

Furthermore, nucleotide sequencing of interpopulation COI gene and GenBank (MW574376.1) found 4 different types of amino acids (Table 6). These variations can be used as genetic markers for *M. maleo* in 3 population areas. Variations in nucleotide substitution at codons 1 and 2 have provided non-synonymous AA substitutions. These substitutions occur at site 2, T→I; site 5, Y→N, and site 262, W>R. These substitutions occurred between GenBank sequences and samples from North, Southeast and Central Sulawesi. On the other hand, at site 10 (H→N), amino acid mutation only occurred in populations from North Sulawesi. Populations from Southeast Sulawesi and Central Sulawesi did not have amino acid mutations. In other words, it is identical to the amino acid in the GenBank standard (MW574376.1).

Table 5. Codon sites mutation in the *Macrocephalon maleo* COI gene

Population and accession number	Codon site			
	2 (5)	**5 (13)**	**10 (28)**	**262 (790)**
MW574376.1	ACT	TAC	CAT	TGG
North Sulawesi	. T .	A. .	A. .	C. .
Southeast Sulawesi	. T .	A. .	. . .	C. .
Central Sulawesi	. T .	A. .	. . .	C. .

Note: Dot symbol (.) is an identical sequence to *Macrocephalon maleo* from Genbank (MW574376.1). The number in parentheses ( ) is the nucleotide site sequence of the *Macrocephalon maleo* COI gene. Codon site: A: Adenine; T: Thymine; G: Guanine; C: Cytosine

Table 6. Amino acids mutation in the *Macrocephalon maleo* COI gene

Population and accession number	Amino acid site			
	2	**5**	**10**	**262**
MW574376.1	T	Y	H	W
North Sulawesi	**I**	**N**	**N**	**R**
Southeast Sulawesi	**I**	**N**	**.**	**R**
Central Sulawesi	**I**	**N**	**.**	**R**

Note: Dot symbol (.) is an identical sequence to *Macrocephalon maleo* from Genbank (MW574376.1). Bold and underlined are non-synonymous changes. Amino acid: H: Histidine; I: Isoleucine; N: Asparagine; R: Arginine; T: Threonine; W: Tryptophan; Y: Tyrosine

Variations in nucleotide bases and specific amino acids in the *M. maleo* interpopulation are suspected to be influenced by environmental conditions. Existing individuals of *M. maleo* must be able to adapt to environmental changes. Maharjan and Ferenci (2018) explained that environmental factors such as habitat, food availability, and temperature could affect physiological and genetic adaptation. Li and Graur (1991) explained that synonymous amino acids are coded by three different codons but have the same type of amino acid and are generally located at the third codon position. Conversely, non-synonymous amino acids are arranged by 3 different codons and form different amino acids.

The COI gene sequence data for *M. maleo* has been presented in GenBank, but in another study, the data which were used as a comparison in data analysis were not explained in detail about the sampling location and the number of samples of *M. maleo*. However, the results of the reconstruction of the phylogenetic tree provide a strong assumption that the sampling locations used for comparison in this study have a closer kinship with populations from Southeast Sulawesi and Central Sulawesi. The results of this study can provide stronger evidence about the genetic character of the Maleo Senkawor because it can represent the genetic characteristics of the Maleo Senkawor population in Sulawesi, especially based on the COI gene.

Genetic distance

Intraspecies genetic distance based on the COI *M. maleo* gene sequence in 3 populations (North Sulawesi, Southeast Sulawesi, and Central Sulawesi) had an average value of 0.000-0.001 (<0.1%). The genetic distance between *M. maleo*, which was collected from population locations and *M. maleo* from GenBank (MW574376.1) was 0.004-0.005 (0.4-0.5%). The genetic distance between *M. maleo* and *A. lathami* (MN356204.1) was 0.145-0.150 (14.5-15%). Further information can be seen in Table 7.

Similar results were also found in the mtDNA COI gene in eagles (*Haliaeetus leucogaster*, *Haliastur indus*, *Spilornis cheela*, *Nisaetus bartelsi*, *Nisaetus cirrhatus*, *Accipiter virgatus*) with a genetic distance of 0-0.03% (Zein 2018) and a genetic distance in intraspecific birds in Chile of 0.3% (Colihueque et al. 2021). The genetic distance based on intraspecificity in birds of the *Picoides* genus was 0.41% and in the *Dendrocopos* genus was 0.51% (Huang et al. 2015).

Meanwhile, the genetic distance based on AA from the COI gene sequence of *M. maleo* showed a relatively similar pattern with its nucleotide sequence. The population from North Sulawesi, Southeast Sulawesi, and Central Sulawesi had an average value of genetic distance 0.000-0.004 (<0.4%). *Macrocephalon maleo* population from 3 study locations had a genetic distance of 0.012-0.015 (1.2-1.5%) compared to *M. maleo* GenBank (MW574376.1). The genetic distance between *M. maleo* and *A. lathami* (MN356204.1) was 0.358-0.369 (35.8-36.9%). *Alectura lathami*, in this analysis, acted as an outgroup. Further information can be seen in Table 8.

The low genetic variation in the *M. maleo* species was suspected to be caused by two factors. First, the presence of the same species' origin caused ancestor genes to be still inherited and, second, the small population size could also reduce genetic variation (Fraser 2017; Linløkken 2018; Saputra and Yuda 2020). In addition, it was hypothesized that endangered species had lower genetic diversity than non-threatened species (Willoughby et al. 2015; Kleinhans and Willows-Munro 2019). Thus, the partial COI (799 bp) gene of *M. maleo* could only be used as a DNA barcode. This was in accordance with Huang and Tu (2016) and Krebs et al. (2017), that explained that the COI gene can be used as a rapid and accurate marker for species identification and phylogeny.

Phylogenetic tree construction

Based on the phylogenetic tree construction using nucleotides and amino acids, *M. maleo* from 3 populations formed the same group (ingroup species). On the other hand, *A. lathami* (MW574376.1) acts as outgroup species (Figure 3).

Table 7. The average genetic distance of *Macrocephalon maleo* interpopulation based on COI gene DNA sequences

Location	[1]	[2]	[3]	[4]	[5]
[1] *Macrocephalon maleo* (North Sulawesi)	0.000*				
[2] *Macrocephalon maleo* (Southeast Sulawesi)	0.001	0.000*			
[3] *Macrocephalon maleo* (Central Sulawesi)	0.001	0.000	0.000*		
[4] *Macrocephalon maleo* (MW574376.1)	0.005	0.004	0.004	0.000*	
[5] *Alectura lathami* (MN356204.1)	0.147	0.145	0.145	0.150	0.000*

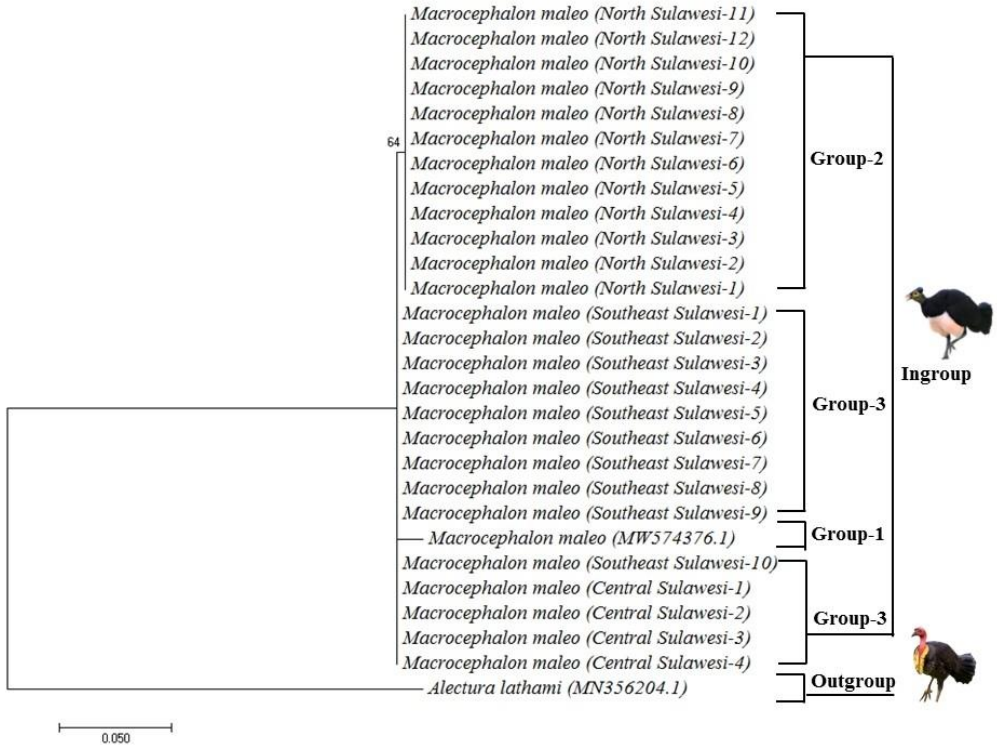
Note: (*) Genetic distance between individuals in the population (intraspecies)

Table 8. The average genetic distance of *Macrocephalon maleo* interpopulation based on amino acids

Location	[1]	[2]	[3]	[4]	[5]
[1] *Macrocephalon maleo* (North Sulawesi)	0.000*				
[2] *Macrocephalon maleo* (Southeast Sulawesi)	0.004	0.000*			
[3] *Macrocephalon maleo* (Central Sulawesi)	0.004	0.000	0.000*		
[4] *Macrocephalon maleo* (MW574376.1)	0.015	0.012	0.012	0.000*	
[5] *Alectura lathami* (MN356204.1)	0.362	0.358	0.358	0.369	0.000*

Note: (*) Genetic distance between individuals in the population (intraspecies)

A



B

Figure 3. The relationship between populations of *Macrocephalon maleo* based on neighbor-joining (NJ) 1000 bootstrap repetitions using the Kimura 2-parameter model (DNA sequence) and the p-distance (amino acid) model for COI gene markers. A. Phylogenetic tree construction based on mtDNA sequences; B. Phylogenetic tree construction based on amino acids

The results of the reconstruction of the *M. maleo* phylogenetic tree using the COI gene showed that the 3 study population locations formed the same group (ingroup species). Meanwhile, the *A. lathami* (MW574376.1) species acted as an outgroup species from *M. maleo*. Although the *M. maleo* forms the same group, each has created a different haplotype. This was suspected to be caused by individuals in each population experiencing geographic isolation. Populations that were geographically isolated over time will have separate genetic patterns (Zhang et al. 2002; Worsham et al. 2017). In addition, the geological activity of Sulawesi Island is suspected to be correlated with population scattering. This event formed geographic isolation and reproductive isolation for the species *M. maleo* (Dinca et al. 2013; Ishikawa et al. 2013; Gebiola et al. 2016; Paterson 2016). Moss and Wilson (1998) explained that about 60-70 million years ago, West Sulawesi separated from Kalimantan, and about 15 million years ago, East Sulawesi separated from Irian. East Sulawesi then moved west and collided with the West Sulawesi fragment, causing the fragment to veer and the northern peninsula to rotate nearly 90 degrees to its current position. Isolation results in the species living on an island evolving with minimal interaction with other populations (Beehler 2020).

Conservation implications

Based on the genetic information of *M. maleo* in 3 populations (North Sulawesi, Southeast Sulawesi, and Central Sulawesi), each population produced a different haplotype. These haplotype differences can be used as characterizing markers in each population. Furthermore, the conservation of *M. maleo* from these 3 populations was better treated as a separate group based on the mitochondrial DNA COI gene. If considered in the same group, this has the potential to eliminate the original gene in each population. In addition, it is also suspected that it can cause impacts such as disease, decreased fitness, and other similar things (Pekkala et al. 2014; Hedrick and Garcia-Dorado 2016; Harrisson et al. 2019).

In conclusion, analysis of the COI *M. maleo* gene from 3 population locations (North Sulawesi, Southeast Sulawesi, and Central Sulawesi) showed that they came from the same species. The relationship between the sample used for comparison with the population from Southeast Sulawesi and Central Sulawesi is close. However, the sample had 4 specific nucleotides (SNPs) at sites 5, 13, 28, and 790. Site 28 is a specific site as a marker for the maleo population from North Sulawesi. The sample also had different amino acids at AA sites 2, 5, 10, and 262.

Macrocephalon maleo from North Sulawesi, Southeast Sulawesi, and Central Sulawesi produced a different haplotype. These differences can be used as characterizing markers in each population. Furthermore, the conservation of *M. maleo* from these 3 populations was better treated as a separate group based on the mitochondrial DNA COI gene.

Molecular research using the COI mtDNA gene is suitable for use in endangered wildlife populations. Results can be obtained quickly and accurately, especially in the identification and phylogenetic aspects.

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