

## Haplotype diversity of Pangolin (*Manis javanica* Desmarest, 1822) based on the COI gene

Niken Februani, Djong Hon Tjong, Wilson Novarino, Mansyurdin, Syaifullah and Dewi Imelda Roesma \*

Biology Department, Faculty of Mathematics and Natural Science, Andalas University, 25163 Padang, West Sumatra, Indonesia.

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### Abstract

The Pangolin (*Manis javanica*) is a unique scaly mammal with the IUCN status as a critically endangered species. In Indonesia, *M. javanica* is distributed in Java, Sumatra, and Kalimantan. The population of *M. javanica* has been declined due to the high poaching. *M. javanica* has been reported present in some locations in West Sumatra however, there is no biological data, especially molecular data. Therefore, it is necessary to conduct the haplotype diversity study of *M. javanica* using the COI gene. Hair samples of *M. javanica* were collected during the release of *M. javanica* in the Biology Education and Research Forest (HPPB) of Andalas University. The result of the study was obtained 601 bp of the COI gene for analysis. Two haplogroups were obtained from five sequences in West Sumatra and 15 Genbank sequences. The value of haplotype diversity was 0.995. The value of nucleotide diversity ( $\pi$ ) in the haplogroup ranged from 0.042 to 0.047. The value of nucleotide diversity ( $\pi$ ) between haplogroups ranged from 0.0148 to 0.0161. Overall, the values indicate the low genetic diversity of pangolin (*M. javanica*). Therefore, it is necessary to maintain and increase the genetic diversity of *M. javanica* within haplogroups.

**Keywords:** COI gene; Conservation; Haplotype diversity; *Manis javanica*; Nucleotide diversity

### 1. Introduction

The Pangolin (*Manis javanica* Desmarest, 1822) is one of the unique scaly mammal species of the four species of the Manidae family. *M. javanica* is distributed in Indonesia with a distribution area covering Sumatra, Java, Kalimantan and surrounding islands [1]. Currently, the population of *M. javanica* has declined [2]. One of the factors causing this population decline is poaching due to the high demand for *M. javanica* meat and scales. Based on data between 2010-2015 [3], there have been 111 seizures with an estimated 32,632 *M. javanica* in the form of whole bodies, meat, scales and skin. [4] state that population declines can limit gene flow, increase inbreeding, and reduce the genetic diversity.

Genetic diversity is a difference that occurs in the organism's genome, either in nucleotide bases, alleles, genes or chromosomes [5]. Information on genetic diversity is needed to determine appropriate conservation status and strategies [6,7]. One of the parameters in genetic diversity analysis is haplotype diversity. Haplotypes are single-base variations in DNA sequences among individuals [8]. Haplotype diversity can be analyzed with molecular markers, one of them is the Cytochrome Oxidase Subunit I (COI) gene.

The COI gene is one of the mitochondrial DNA genes with many conserved and mutation regions to identify organisms up to the species level [9,10]. In addition, the COI gene has been used in haplotype network analysis and phylogenetic tree reconstruction in confiscated *M. javanica* samples [11,12].

\* Corresponding author: Dewi Imelda Roesma.

Molecular studies of *M. javanica* using the COI gene have previously been conducted by [12] on 77 confiscated samples. Among the confiscated samples of known origin are those from Sibolga (North Sumatra), Bengkulu (South Sumatra), and East Kalimantan. The study reported that each population had different haplotypes. In addition, *M. javanica* was also found in West Sumatra [13]. However, the available information is only limited to their presence, and there is no biological data, especially molecular data. The haplotype diversity information is any of the required molecular data to determine the conservation strategies of *M. javanica* in West Sumatra (Centra Sumatra)[6,7]. Haplotype diversity information of *M. javanica* in West Sumatra will be useful as a reference to determine the status and conservation strategies of *M. javanica* in Indonesia.

## 2. Materials and methods

### 2.1. Sample sources

The samples used in this study consisted of five hair samples from five individuals of *M. javanica*. Hair samples were obtained from the release of *M. javanica* in the HPPB (Biology Education and Research Forest) with the West Sumatra Natural Resources Conservation Center (BKSDA).

**Table 1** List of *M. javanica* samples and their origin

No	Location	Sample Code
1	Dadok Tunggul Hitam, West Sumatra	MJ1
2	Bukittinggi, West Sumatra	MJ2
3	Sungai Saria, West Sumatra	MJ3
4	Padusunan Pariaman, West Sumatra	MJ4
5	Limau Manis, West Sumatra	MJ5

### 2.2. DNA Isolation and Amplification

DNA isolation stages follow the GeneAll Exgene Genomic DNA Micro Kit protocol. The results of DNA isolation were then electrophoresed using a 1.2% agarose gel. Furthermore, DNA amplification was carried out using a PCR reaction mixture with a total volume of 25 µl consisting of Supermix Biotline 12.5µl, Nuclease Freewater 2.5 µl, forward primer 2µl, reverse primer 2µl, and DNA isolates 6µl. Amplification DNA using primer pair pangolin-COI-HFZ3 (forward) and pangolin-COI-HZF3 (reverse) [11]. The PCR process began with pre-denaturation at 94°C for 1 minute, followed by denaturation at 94°C for 30 seconds, annealing at 52°C for 20 seconds and elongation at 72°C for 50 seconds. In the last cycle, the final extension was at 72°C for 2 minutes. The amplification process with a PCR machine runs for 35 cycles. PCR products were electrophoresed using a 2% agarose gel. Then, PCR products were sent to Firstbase Company Malaysia for sequencing.

### 2.3. DNA Sequence Analysis

The contiguous DNA sequences of *M. javanica* were then BLAST and compared with sequences downloaded from the NCBI GenBank (Table 2).

**Table 2** List of comparison sequences species from NCBI data

No	GenBank Accession	Location	Authors
1	MT035778.1	Sibolga, North Sumatra 1	[12]
2	MT035777.1	Sibolga, North Sumatra 2	[12]
3	MT035776.1	Sibolga, North Sumatra 3	[12]
4	MT035775.1	Sibolga, North Sumatra 4	[12]
5	MT035754.1	Sibolga, North Sumatra 5	[12]

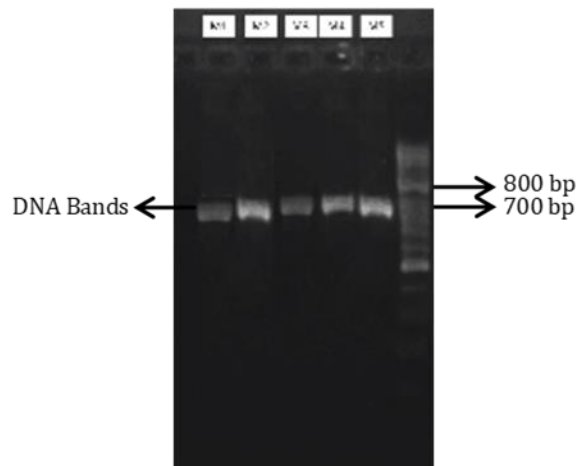
6	MT035753.1	Sibolga, North Sumatra 6	[12]
7	MT035764.1	Bengkulu 1	[12]
8	MT035763.1	Bengkulu 2	[12]
9	MT035762.1	Bengkulu 3	[12]
10	MT035761.1	Bengkulu 4	[12]
11	MT035752.1	East Kalimantan 1	[12]
12	MT035751.1	East Kalimantan 2	[12]
13	MT035750.1	East Kalimantan 3	[12]
14	MT035749.1	East Kalimantan 4	[12]
15	MT035748.1	East Kalimantan 5	[12]

All DNA sequences were aligned using the CLUSTAL X program [14]. Polymorphism sequence analysis was performed with the DNA sequence polymorphism 5.10 program [15] and Haplotype network analysis was performed with NETWORK version 10.2.0.0 [16].

### 3. Results and Discussion

#### 3.1. Amplification of pangolin (*M. javanica*) DNA

Five *M. javanica* samples were successfully amplified and visualized on a 2% agarose gel. A clear band with a fragment length of 700-800 bp indicates that the amplification results are in accordance with the primer target [11], which is around 793 bp.



**Figure 1** Electrophoresis of COI gene amplification results in *M. javanica* using 2% agarose gel.

#### 3.2. BLAST and Alignment Analysis

The BLAST results showed 98–99% similarity of samples sequences with other *M. javanica* in Genbank sequences. The position of the COI gene sequence (601 bp) is located at position 5415-6015 bp from the complete genome of mitochondrial DNA. The study [17] reported the position of the COI gene of *M. javanica* in the complete genome of mitochondrial DNA at position 5326-6876 bp. Out of 20 sequences, there are 573 bp (95.34%) as conserved sites, 28 bp (4.65%) as variable sites, 16 bp (2.66%) as parsimony sites, and 12 bp (1.99%) as singleton sites. The nucleotide base composition was 25.2% A (Adenine), 18.2% G (Guanine), 27.1% T (Thymine), and 29.4% C (Cytosine). Adenine +

Thymine (AT) nucleotide base was 52.3%, Guanine + Cytosine (GC) was 47.6%. The study of [17] reported the AT content in the Pholidota order is higher than the GC content.

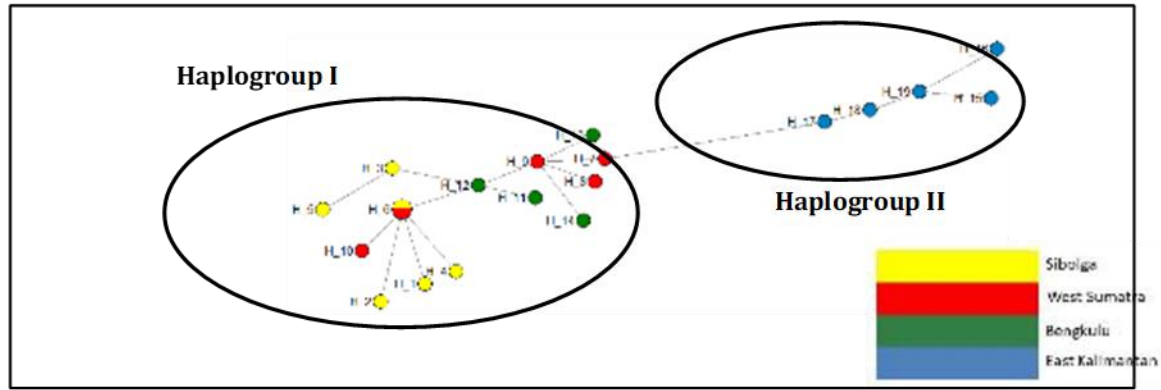
### 3.3. Haplotype and haplotype network analysis

The alignment of all sequences of the *M. javanica* West Sumatra population and Genbank sequences were used for haplotype analysis. Based on the analysis of polymorphic sequences using DNA Sequence Polymorphism 5.10 [15], it can be concluded that there are 19 haplotypes from 20 individuals with a sequence length of 601 bp (Table 3). Based on haplotype network analysis, 19 haplotypes from 20 individuals clustered into two main haplogroups. Haplogroup I consists of individuals from the Sumatra population. Haplogroup II consists of individuals from the Kalimantan population. The formation of the two haplogroups is due to a physical barrier in the form of the sea, thus inhibiting gene flow between populations. [18] stated that geographically isolated species cause high genetic differences due to the absence of gene flow.

**Table 3** Haplotypes of *M. javanica* with COI gene

No	Haplotype	Location
1	Haplotype 1	Sibolga, North Sumatra 1
2	Haplotype 2	Sibolga, North Sumatra 2
3	Haplotype 3	Sibolga, North Sumatra 3
4	Haplotype 4	Sibolga, North Sumatra 4
5	Haplotype 5	Sibolga, North Sumatra 5
6	Haplotype 6	Sibolga, North Sumatra 6; Limau Manis, West Sumatra 5
7	Haplotype 7	Dadok Tunggul Hitam, West Sumatra 1
8	Haplotype 8	Bukittinggi, West Sumatra 2
9	Haplotype 9	Sungai Sariak, West Sumatra 3
10	Haplotype 10	Padusunan Pariaman, West Sumatra 4
11	Haplotype 11	Bengkulu 1
12	Haplotype 12	Bengkulu 2
13	Haplotype 13	Bengkulu 3
14	Haplotype 14	Bengkulu 4
15	Haplotype 15	East Kalimantan 1
16	Haplotype 16	East Kalimantan 2
17	Haplotype 17	East Kalimantan 3
18	Haplotype 18	East Kalimantan 4
19	Haplotype 19	East Kalimantan 5

Haplotype network analysis showed *M. javanica* from West Sumatran population grouped in haplogroup I. The haplotype distribution in haplogroup I is shown in Figure 2. The figure shows that there is not any specific grouping in each Sumatran population. The haplotype distribution shows the sharing of haplotype 6 (H6) by individuals from the Sibolga population (Northern Sumatra) with the West Sumatra population (Central Sumatra) (Table 3). The sharing of the same haplotypes between two populations of *M. javanica* showed they originated from the same ancestor [19].



**Figure 2** Haplotype network of *M. javanica* based on COI gene

### 3.4. Haplotype Diversity (Hd) and Nucleotide Diversity ( $\pi$ )

The analysis of haplotype diversity (Hd) and nucleotide diversity ( $\pi$ ) was conducted using the DNA Sequence Polymorphism (DNASP) v 5.10 software (Table 4 and Table 5).

**Table 4** Haplotype diversity (Hd) and nucleotide diversity ( $\pi$ ) of *M. javanica* based on COI gene

Location	n	Hn	Hd	$\pi$
Sibolga	6	6	1,0000	0,0037
West Sumatra	5	5	1,0000	0,0040
Bengkulu	4	4	1,0000	0,0044
East Kalimantan	5	5	1,0000	0,0037
Total	20	19	0,9947	0,0124

Note: n = number of samples obtained, Hn = number of haplotypes, Hd = haplotype diversity,  $\pi$  = nucleotide diversity

**Table 5** Nucleotide diversity ( $\pi$ ) values between *M. javanica*

No	Location	1	2	3	4
1	North Sumatra	-	-	-	-
2	West Sumatra	0,0042	-	-	-
3	Bengkulu	0,0047	0,0042	-	-
4	East Kalimantan	0,0151	0,0148	0,0161	-

The haplotype diversity of *M. javanica* is 0,995 (Table 4). Overall, the value showed the high haplotype diversity of *M. javanica* populations. [20] stated that the value of haplotype diversity (Hd) is high if it has a range value >0.5 and low if it has a range value <0.5. The nucleotide diversity of *M. javanica* with a sequence length of 601 bp within haplogroups ranged from 0.042 to 0.047, while the nucleotide diversity between haplogroups ranged from 0.0148 to 0.0161. The value showed the low nucleotide diversity within and between haplogroups. The value of nucleotide diversity within haplogroups is inversely proportional to the value of haplotype diversity. [21] stated that a high haplotype diversity with a low nucleotide diversity value indicates that there are few differences in nucleotide bases in each haplotype. The low genetic diversity could threaten the survival ability of *M. javanica* in the haplogroup. Therefore, it is necessary to maintain and increase the genetic diversity of *M. javanica* within haplogroups.

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#### 4. Conclusion

The result showed that based on 601 bp in the COI gene, 19 haplotypes were obtained from five sequences in West Sumatra and 15 Genbank. The value of haplotype diversity and nucleotide diversity indicate the low genetic diversity of pangolin (*M. javanica*).

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#### Compliance with ethical standards

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##### *Disclosure of conflict of interest*

There is no interest in the conflict between the authors of this piece of research work. The authors agreed and assigned in hand to all matter arise to this piece of research work.

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