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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan



Research Article

Phylogenetic Relationships of Sun Bear (*Helarctos malayanus*) Based on Mitochondrial DNA from Sumatra and Other Southeast Asian Regions

Dewi Imelda Roesma, Djong Hon Tjong, Syaifullah, Dyta Rabbani Aidil, Muhammad Ryan Maulana and Viola Mutiara Salis

Department of Biology, Faculty of Mathematics and Natural Science, Andalas University, Padang, West Sumatra 25175, Indonesia

Abstract

Background and Objective: The *Helarctos malayanus* is the sole bear species living in Indonesia (Sumatra and Borneo). The available biological data for sun bears (*H. malayanus*) in Sumatra is limited, especially for morphological and genetic data. A morphological approach is difficult to do. Therefore, a molecular approach is the most likely choice. Phylogenetic analysis was carried out on *H. malayanus* in Central Sumatra (Dharmasraya, South Solok and Riau) using the Cytochrome B gene. **Materials and Methods:** Blood samples from three individuals of *H. malayanus* were obtained at the Sumatran Tiger Rehabilitation Center, Dharmasraya. Three *H. malayanus* Central Sumatra sequences and 62 GenBank sequences were used in the analysis. The DNA sequences were analyzed using the DNA Star, AliView, Bioedit, DNA SP, haplotype network, IQ Tree and MEGA software. **Results:** Forty-one haplotypes were identified in 65 sequences, with 17 haplotypes belonging to *H. malayanus*. Haplotype network analysis divides *H. malayanus* into Haplogroup I (Sundaland) and Haplogroup II (Mainland). All individuals of *H. malayanus* in Central Sumatra have the same haplotype as Peninsular Malaysia sequence. The sun bear (*H. malayanus*) has a monophyletic relationship with other bear species. The *H. malayanus* has a higher genetic distance between the two lineages (1.0-2.3%) than the genetic distance within the subpopulations of each lineage. **Conclusion:** The study results supported sun bear (*H. malayanus*) divided into two different lineages: Mainland (subcluster 1) and Sundaland (subcluster 2 and 3). The geographic isolation causes the absence of gene flow, which results in high genetic distance between sun bears (*H. malayanus*) in Sundaland and Mainland lineages.

Key words: *Helarctos malayanus*, Sumatra, sun bear, cytochrome b, Sundaland, Mainland

Citation: Roesma, D.I., D.H. Tjong, Syaifullah, D.R. Aidil, M.R. Maulana and V.M. Salis, 2023. Phylogenetic relationships of sun bear (*Helarctos malayanus*) based on mitochondrial DNA from Sumatra and other Southeast Asian Regions. Pak. J. Biol. Sci., 26: 615-627.

Corresponding Author: Dewi Imelda Roesma, Department of Biology, Faculty of Mathematics and Natural Science, Andalas University, Padang, West Sumatra 25175, Indonesia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Carnivora are an order of mammal class in which almost all species have carnivorous diets (meat eaters)¹. One of the families in Carnivora is the Ursidae, a member of the Caniformia suborder². Ursidae is a family of bears that have adapted to various climates, extending across the Northern and Southern Hemispheres^{2,3}. Despite belonging to the Carnivora, most bears are omnivores with different diets^{1,4}. Since prehistoric times, bears have hunted for various uses such as food, entertainment, folk medicine, mythology and other cultural aspects^{5,6}. Currently, only eight species of bears are spread across North and South America, Europe and Asia^{2,7}. All species are included in three subfamilies: Ailuropodinae (giant panda), Tremarctinae (spectacled bear) and Ursinae (sloth bear, sun bear, asian black bear, american black bear, brown bear and polar bear)^{2,7}. Six of eight bear species are listed as vulnerable/endangered in the IUCN Red List.

The sun bear is the sole bear species living in Indonesia and the smallest bear in the world^{8,9}. Based on its historical distribution (within 500 years), the sun bear extended across the tropical rainforest areas of much of Southeast Asia, from Indonesia to Northern China and India. Indonesia's sun bear range extends South and East (Sumatra and Borneo), known as the Sundaic Region¹⁰. The scientific name sun bear was first established as "*Ursus malayanus*" by Raffles in 1821, with the type locality in Sumatra. Then, Horsfield (1825) gave the name "*Helarctos euryspilus*" for the type locality in Borneo (Kalimantan). The difference in scientific names is due to the differences in body size between Borneo and Sumatran sun bears. However, due to the lack of comparative data other than body size, the scientific name for sun bears from all locations is determined as *H. malayanus* (Raffles, 1821)^{11,12}. The size difference becomes the basis for describing two subspecies of *H. malayanus* (Horsfield, 1825): *H. m. euryspilus* only for Borneo (Kalimantan) and *H. m. malayanus* for other Southeast Asia regions (small parts of China, India, Malaysia and Sumatra)¹². *Helarctos malayanus* is a bear species with a vulnerable status that has declined populations due to deforestation, habitat loss and fragmentation, poaching and illegal trade in body parts with high market value^{6,13-17}. The continuously declining population will significantly impact its genetic structure, demography structure and viability^{6,14-16,18}.

Sun bears are often referred to as the least understood or least known bears of other bears due to the limited information and studies on the sun bear population¹⁹. Increasing scientific knowledge regarding the ecology, population distribution, genetics and impact of threats to sun

bear is urgent. Since the 2000s, studies on several aspects, such as the ecology, distribution, disease and genetics of sun bear, have been conducted and still need to be expanded in some aspects. Some studies have been conducted include food habits, home range, movement and activity patterns, bedding site and impacts of El Niño-related drought and forest fires on sun bears in Borneo^{9,20-23}, distribution of *H. malayanus*^{24,25}, diseases in sun bears²⁶⁻²⁸, habitat, population size and conservation for the sun bear^{29,30}, exploring potential range connectivity of sun bear³¹ and genetic studies of *H. malayanus*³²⁻³⁵.

A study visual analysis to assess potential range connectivity using tree cover in the sun bear by Scotson³¹ identified the seven potential subpopulations of sun bears: (i) Northern Mainland, (ii) Central Myanmar, (iii) Central South East Asia, (iv) South-Central South East Asia, (v) Thai-Malay peninsula, (vi) Sumatra and (vii) Borneo (Kalimantan). Habitat fragmentation, high human influence and road construction are potential barriers to movement between populations that cause isolated populations³¹. The isolation also impacts morphological and genetic variations between sub-populations. Although Sumatra is a type locality of the sun bear, the biological data available for the Sumatran population is limited, especially morphological and genetic data. Based on the investigations of sun bear distribution in Sumatra, they are present in several areas in Central Sumatra and protected at the Sumatran Tiger Rehabilitation Center (PRHSD) in Dharmasraya, West Sumatra. Therefore, providing data and information on the Sumatran sun bear is becoming essential. The morphological approach is challenging and the molecular approach is the only way. This study aimed to determine the phylogenetic relationship and haplotype diversity of sun bear (*H. malayanus*) in Sumatra and other Southeast Asian regions using a mitochondrial DNA marker (cytochrome b). The result will be helpful as a reference for further study in another aspect of the *H. malayanus*.

MATERIALS AND METHODS

Study area: Sun bear (*H. malayanus*) individuals were collected in November, 2022 and housed at the Arsari Foundation, Sumatran Tiger Rehabilitation Center (PRHSD), Dharmasraya, West Sumatra. Samples originate from three regions in Central Sumatra (Dharmasraya, South Solok and Riau). All the molecular analyses were performed from April, 2023 to July, 2023 in the Genetic and Biomolecular Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences Andalas University, Padang, Indonesia.

Sample collection: Blood samples from three *H. malayanus* individuals were taken by doctors at PRHSD Sumatra. Blood samples were collected before being transferred to the Sumatra Wildlife Center-Indonesian Animal Network Foundation (SWC-YJSI). Blood samples were obtained utilizing EDTA and transferred into 1.5 mL Vocolab tubes. Samples were stored at cold temperatures and taken to the Genetics and Biomolecular Laboratory Andalas University, for further analysis.

DNA isolation, amplification DNA and sequencing of DNA:

The DNA isolation followed the GeneAll Exgene Genomic DNA mini kit protocol for blood sample isolation. The DNA isolate's quality was assessed by electrophoresis, employing a 1.2% agarose gel in a TBE solution. The electrophoresis results were checked using a documentation gel with a UV illuminator. Amplification of the Cyt b mtDNA gene in *H. malayanus* was carried out using design primer, Hm cyt b F: GGCGTGATGAAACTTCGGAT for the forward primer and Hm cyt b R: GGGTGCTCAATGGGATTGTC for the reverse primer. The process of DNA amplification was conducted using a 30 µL solution comprising 15 µL Bioline Supermix solution, 9 µL ddH₂O, one µL of forward primer, one µL of reverse primer and 4 µL of DNA isolate. The Polymerase Chain Reaction (PCR) process takes place in four stages: Pre-denaturation at 95°C for 5 min followed by 35 cycles for denaturation at 94°C for 60 sec, annealing at 54°C for 60 sec and extension at 72°C for 60 sec. The final extension occurs at 72°C for 7 min while the PCR results are preserved at 4°C. The PCR products were purified at the Genetic Science Laboratory and sent to First Base Malaysia for sequencing.

Statistical analysis: All sequencing results were contig (forward and reverse sequences) using the DNA star software³⁶. The contig results were checked for sequence similarity using the BLAST³⁷. Sixty-two comparison sequences were obtained from GenBank, NCBI, to align with three *H. malayanus* sequences from Central Sumatra using the AliView software³⁸ and checked using the Bioedit. The amino acid of the DNA sequence was checked using the DNA to protein translation. Polymorphism sequence data (haplotype type, haplotype diversity and nucleotide diversity) were analyzed using the DNA SP V 6.0 software³⁹ to assess variations in nucleotide bases. Haplotype network analysis was carried out on three samples of *H. malayanus* from Central Sumatra and 38 comparison sequences (*H. malayanus* GenBank, NCBI) using the haplotype network 10.0 software. The phylogenetic tree was reconstructed using the IQ Tree with the maximum likelihood method with 5000 bootstraps. Genetic distance values were analyzed using the Molecular Evolutionary Genetics Analysis (MEGA) V 11.0 software⁴⁰.

RESULTS

Nucleotide base variations: A total of three sequences of *H. malayanus* collected from Central Sumatra and 62 sequences from GenBank (38 *H. malayanus* sequences from GenBank) were used for analysis. The length of the sequence analyzed was 640 bp. The alignment results showed *H. malayanus* is located at positions 15,148-15,843 within the complete mitochondrial genome. Meanwhile, all alignment sequences are located at positions 144-783 bp in the complete Cyt b gene. Out of the 640 bp analyzed, there were 372 bp (58.12%) conserved sites and 268 bp (41.88%) variable sites. Out of the 268 bp variable sites, there are 229 bp (35.78%) parsimony sites and 39 bp (6.10%) singleton sites. The nucleotide base composition of the Cyt b gene for *H. malayanus* is A (adenine) 27.3%, T (thymine) 27.6%, G (guanine) 15.3% and C (cytosine) 29.8%. The proportion of the nucleotide base adenine+thymine (A+T) is 54.9%, whereas the proportion of the nucleotide base guanine+cytosine (G+C) is 45.1%. There are 41 *H. malayanus* sequences from seven sub-populations: Cambodia, Sumatra (including three Sumatran sequences in this study), Peninsular Malaysia, Borneo, Thailand, China and the USA.

A total of 29 variations of nucleotide bases were found in the *H. malayanus* sequences (Table 1). The *H. malayanus* Peninsular Malaysia MN807949.1 is a reference sequence for other *H. malayanus* sequences. Within the Peninsular Malaysia subpopulation, there is a single nucleotide base change in *H. malayanus* Peninsular Malaysia OQ564458.1 at the 556th base (T→A). Within the Sumatran subpopulation, nine nucleotide bases differ from the reference sequence in five Sumatra GenBank sequences (Table 1). Meanwhile, the three *H. malayanus* sequences in Central Sumatra didn't have different variations and had similarities in all nucleotide bases (100%) with the reference sequence (*H. malayanus* Peninsular Malaysia MN807949.1). The Borneo subpopulation has only one sequence, with four different nucleotide bases from the reference sequence. In Thailand's subpopulation, three out of four sequences have different patterns of nucleotide base variation. Interestingly, *H. malayanus* Thailand OQ564463.1 has the same nucleotide base variation as *H. malayanus* Borneo. The results showed similar nucleotide base patterns in *H. malayanus* based on geographic region. Sumatra, Peninsular Malaysia and Borneo subpopulations have almost the same pattern of nucleotide base variations. These subpopulations are included in the Sundaland group. However, several individuals from Cambodia, Thailand and unknown origin also have the same nucleotide base variations as the Sundaland subpopulations.

Table 1: Variable site of nucleotide base of *H. malayanus*

		1	1	1	2	2	2	2	2	3	3	3	3	4	4	4	4	4	4	5	5	5	5	5	5	6				
		3	5	2	6	7	0	2	5	7	8	3	5	7	7	1	1	2	2	3	6	6	1	3	4	5	7	8	8	1
Species		1	6	4	0	5	5	5	6	1	6	1	5	3	6	5	9	4	8	9	6	9	7	8	1	6	5	0	6	9
MN807949.1 <i>H. malayanus</i> Peninsular Malaysia	C	A	G	C	G	C	T	A	A	G	G	G	A	A	G	G	C	G	G	A	G	T	G	C	T	A	T	T	C	
OQ564459.1 <i>H. malayanus</i> Peninsular Malaysia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<i>H. malayanus</i> Central Sumatra 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<i>H. malayanus</i> Central Sumatra 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<i>H. malayanus</i> Central Sumatra 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
OQ564487.1 <i>H. malayanus</i> Sumatra	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
OQ564486.1 <i>H. malayanus</i> Sumatra	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
OQ564489.1 <i>H. malayanus</i> Unknown	.	G	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
OQ564469.1 <i>H. malayanus</i> Cambodia	.	G	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
OQ564488.1 <i>H. malayanus</i> Unknown	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	C	.	
OQ564462.1 <i>H. malayanus</i> Unknown	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	C	.	
OQ564464.1 <i>H. malayanus</i> Sumatra	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	C	T	
OQ564458.1 <i>H. malayanus</i> Peninsular Malaysia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	
OQ564468.1 <i>H. malayanus</i> Thailand	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	A	.	.	.	.	.	
OQ564466.1 <i>H. malayanus</i> Borneo	T	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	
OQ564463.1 <i>H. malayanus</i> Thailand	T	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	
OQ564465.1 <i>H. malayanus</i> Sumatra	T	.	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	A	.	.	.	.	.	
OQ564461.1 <i>H. malayanus</i> Sumatra	T	.	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	A	.	.	.	.	.	
OQ564480.1 <i>H. malayanus</i> Cambodia	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	A	G	.	.	.	.	
OQ564484.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564476.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
FM177765.1 <i>H. malayanus</i> USA	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564467.1 <i>H. malayanus</i> Thailand	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564460.1 <i>H. malayanus</i> Thailand	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564483.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564482.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564481.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564477.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564475.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564473.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564478.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564470.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	.	A	.	.	.	.	.	A	.	.	.	.	.	.	A	.	A	.	C	.	.	
EF196664.1 <i>H. malayanus</i> China	.	.	.	T	.	T	.	.	G	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
NC 009968.1 <i>H. malayanus</i> China	.	.	.	T	.	T	.	.	G	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
MG366865.1 <i>H. malayanus</i> Unknown	.	.	.	T	.	T	.	.	G	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564471.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	G	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564474.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	G	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564479.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	G	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564472.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	G	A	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
OQ564485.1 <i>H. malayanus</i> Cambodia	.	.	.	T	.	T	.	G	G	A	A	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	
U18899.1 <i>H. malayanus</i> Missouri Zoo (USA)	.	.	.	T	.	T	.	.	G	A	.	A	G	G	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	

Cambodia has the largest subpopulation, consisting of 17 individuals. There are 15 nucleotide bases variation in the Cambodian subpopulation with a reference sequence. There are six patterns of nucleotide base variation in the 17 Cambodian individuals. China and the USA subpopulations also have variations that are almost similar to Cambodia's subpopulation. Cambodia, China and the USA are subpopulations within the geographical region referred to as the Mainland. These subpopulations are included in the Mainland group. However, interestingly there are also two Thailand individuals (OQ564460.1 and OQ564467.1), which have the same variations as the Mainland subpopulations. Overall, there are a total of 11 different variations of nucleotide

bases between the Sundaland and Mainland groups (Table 1). This variation can be a specific base for distinguishing between the Mainland and Sundaland lineages.

Haplotype analysis: Variable base sites are different bases in specific sites that result in individual variations called haplotypes. Haplotype represents the genetic variation of each individual with two or more polymorphic sites in DNA sequences. An analysis of haplotypes was performed using DNA sequence polymorphism software (DNA SP) in 41 *H. malayanus* sequences and 24 other bear sequences. Total of 40 haplotypes were obtained from the 65 sequences analyzed (Table 2). Overall, the haplotype diversity (Hd) and

Table 2: Haplotype data of *H. malayanus* and comparison sequences

Haplotype	Haplotype code	Number	Species
Haplotype 1	H1	5	MN807949.1 <i>H. malayanus</i> Peninsular Malaysia, OQ564459.1 <i>H. malayanus</i> Peninsular Malaysia, <i>H. malayanus</i> Dharmasraya, Central Sumatra 1, <i>H. malayanus</i> Riau, Central Sumatra 2, <i>H. malayanus</i> South Solok, Sumatra 3
Haplotype 2	H2	2	OQ564487.1 <i>H. malayanus</i> Sumatra, OQ564486.1 <i>H. malayanus</i> Sumatra
Haplotype 3	H3	1	OQ564489.1 <i>H. malayanus</i> Unknown
Haplotype 4	H4	1	OQ564469.1 <i>H. malayanus</i> Cambodia
Haplotype 5	H5	2	OQ564488.1 <i>H. malayanus</i> Unknown, OQ564462.1 <i>H. malayanus</i> Unknown
Haplotype 6	H6	1	OQ564464.1 <i>H. malayanus</i> Sumatra
Haplotype 7	H7	1	OQ564458.1 <i>H. malayanus</i> Peninsular Malaysia
Haplotype 8	H8	1	OQ564468.1 <i>H. malayanus</i> Thailand
Haplotype 9	H9	2	OQ564466.1 <i>H. malayanus</i> Borneo, OQ564463.1 <i>H. malayanus</i> Thailand
Haplotype 10	H10	2	OQ564461.1 <i>H. malayanus</i> Sumatra, OQ564465.1 <i>H. malayanus</i> Sumatra
Haplotype 11	H11	1	OQ564480.1 <i>H. malayanus</i> Cambodia
Haplotype 12	H12	12	OQ564484.1 <i>H. malayanus</i> Cambodia, OQ564476.1 <i>H. malayanus</i> Cambodia, FM177765.1 <i>H. malayanus</i> USA, OQ564467.1 <i>H. malayanus</i> Thailand, OQ564460.1 <i>H. malayanus</i> Thailand, OQ564483.1 <i>H. malayanus</i> Cambodia, OQ564482.1 <i>H. malayanus</i> Cambodia, OQ564481.1 <i>H. malayanus</i> Cambodia, OQ564477.1 <i>H. malayanus</i> Cambodia, OQ564475.1 <i>H. malayanus</i> Cambodia, OQ564473.1 <i>H. malayanus</i> Cambodia, OQ564478.1 <i>H. malayanus</i> Cambodia
Haplotype 13	H13	1	OQ564470.1 <i>H. malayanus</i> Cambodia
Haplotype 14	H14	3	EF196664.1 <i>H. malayanus</i> China, NC 009968.1 <i>H. malayanus</i> China, MG366865.1 <i>H. malayanus</i> Cambodia
Haplotype 15	H15	4	OQ564479.1 <i>H. malayanus</i> Cambodia, OQ564472.1 <i>H. malayanus</i> Cambodia, OQ564471.1 <i>H. malayanus</i> Cambodia, OQ564474.1, <i>H. malayanus</i> Cambodia
Haplotype 16	H16	1	OQ564485.1 <i>H. malayanus</i> Cambodia
Haplotype 17	H17	1	U18899.1 <i>H. malayanus</i> Missouri Zoo, USA
Haplotype 18	H18	1	KF184279.1 <i>Ursus americanus</i> Montana, USA
Haplotype 19	H19	1	OQ318943.1 <i>Ursus americanus</i> USA
Haplotype 20	H20	1	OQ318912.1 <i>Ursus americanus</i> USA
Haplotype 21	H21	1	AF007912.1 <i>Ursus americanus kermodei</i> Canada
Haplotype 22	H22	1	EF667005.1 <i>Ursus thibetanus ussuricus</i> Korea
Haplotype 23	H23	1	DQ402478.1 <i>Ursus thibetanus mupinensis</i> China
Haplotype 24	H24	1	MH281753.1 <i>Ursus thibetanus laniger</i> Nepal
Haplotype 25	H25	1	AB020905.1 <i>Ursus arctos</i> Japan
Haplotype 26	H26	2	KF184290.1 <i>Ursus arctos</i> Alaska, U18888.1 <i>Ursus arctos</i> Alaska, USA
Haplotype 27	H27	1	MW991401.1 <i>Ursus arctos</i> Russia
Haplotype 28	H28	1	U18898.1 <i>Ursus maritimus</i> Alaska, USA
Haplotype 29	H29	1	KF184261.1 <i>Ursus maritimus</i> USA
Haplotype 30	H30	1	JX196391.1 <i>Ursus maritimus</i> Svalbard, Norwegia
Haplotype 31	H31	1	U23560.1 <i>Melursus ursinus</i> Missouri Zoo, USA
Haplotype 32	H32	1	MG366862.1 <i>Melursus ursinus</i> India
Haplotype 33	H33	2	MH931229.1 <i>Melursus ursinus</i> India
Haplotype 34	H34	1	MW556430.1 <i>Tremarctos ornatus</i> USA
Haplotype 35	H35	1	FM177764.1 <i>Tremarctos ornatus</i> Oregon, USA
Haplotype 36	H36	1	U23554.1 <i>Tremarctos ornatus</i> Missouri Zoo, USA
Haplotype 37	H37	2	NC 009492.1 <i>Ailuropoda melanoleuca</i> China, EF212882.1 <i>Ailuropoda melanoleuca</i> China
Haplotype 38	H38	1	OL702785.1 <i>Ailuropoda melanoleuca qinlingensis</i> China
Haplotype 39	H39	1	NC 001700.1 <i>Felis catus</i> USA
Haplotype 40	H40	1	OK052506.1 <i>Canis lupus</i> France

nucleotide diversity (π) values for the 65 sequences were $Hd: 0.957 \pm 0.016$ and $\pi: 0.07693 \pm 0.010$, respectively. The total number of base mutations that occurred was 342 mutations, with a transition/transversion bias value of $R = 14,217$. The mutations in all sequences cause the changes in the 53 amino acids.

Seventeen haplotypes belong to the sun bear (*H. malayanus*) and 23 haplotypes belong to other bear sequences. A total of 13 haplotypes belong to species in the *Ursus* genus, three haplotypes belong to species in the *Melursus* genus, three haplotypes belong to species in the

Tremarctos genus, three haplotypes belong to species in the *Ailuropoda* genus and two haplotypes belong to outgroup species (*Felis catus* and *Canis lupus*). The absence of sharing the same haplotype between different species shows the accuracy of different sequences between other species. Haplotype variations belonging to 65 sequences are shown in Table 2. Haplotype variations for *H. malayanus* are visualized in a haplotype network (Fig. 1). The haplotype network visualizes distribution patterns of haplotypes based on mutations in the nucleotide base. The haplotype network parts consist of circle shapes, circle size, mutation numbers,

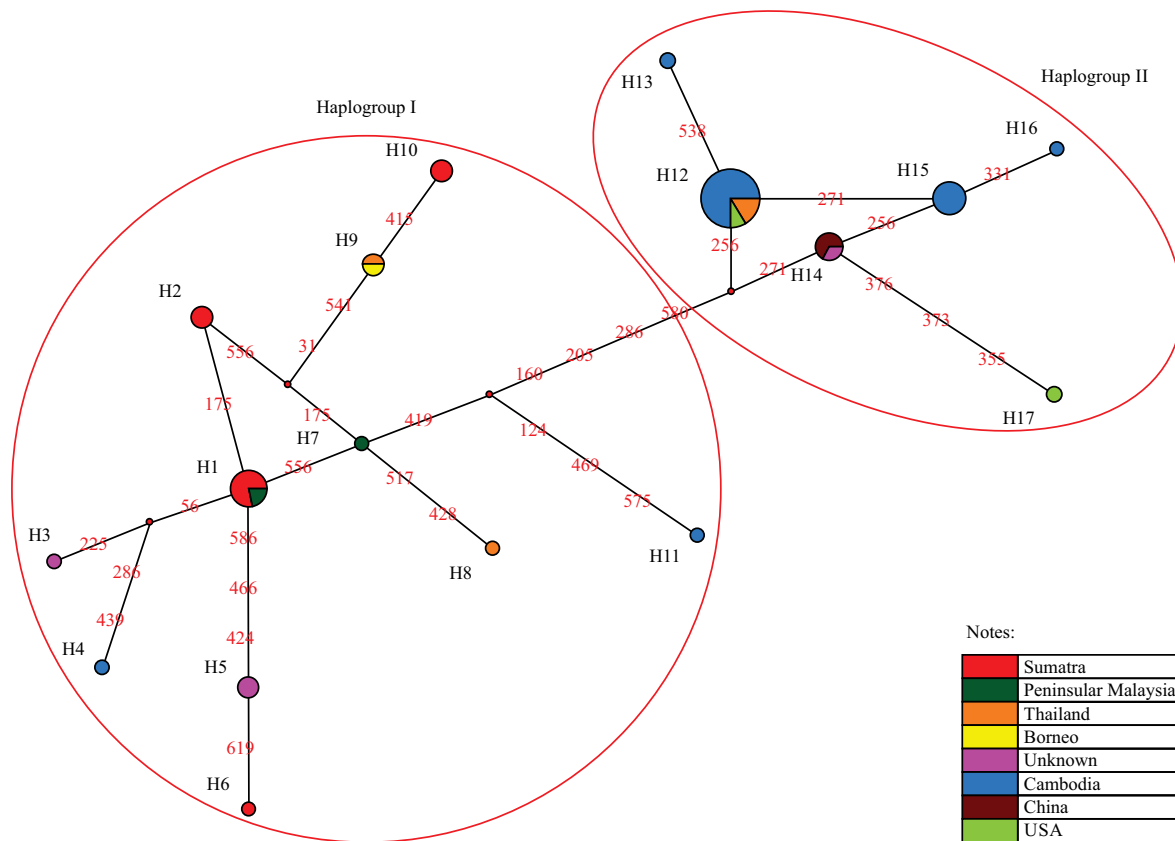


Fig. 1: Haplotype network of *H. malayanus*

branch lines and red dots. The circle shape represents the haplotype type, the circle size represents the number of individuals in the haplotype and the mutation numbers represent base sites mutated. The branch represents the haplotype formation based on the mutated base and the red dot indicates the median vector, which is the meeting point between the haplotypes.

Overall, 29 bases underwent mutations that resulted in 17 haplotypes for *H. malayanus*. Among the 29 nucleotides mutated, 28 bases are transition mutations and one base is transversion mutation. Two types of mutations occur: Silent mutations and missense mutations. Among the 41 *H. malayanus* sequences, five missense mutations cause changes in five amino acids: In the 19th site, the amino acids change from threonine (T) to alanine (A) (codon ACT–GCT), in the 75th site, change from valine to alanine (codon GTT–GCC), in the 140th site, change from valine to isoleucine (GTC–ATC codon), in the 143th site, change from alanine to threonine (codon GCA–ACA) and the 192th site, change from threonine to alanine (codon ACA–GCA). Missense mutations occurred in 26 individuals of *H. malayanus* from several

individuals of Cambodia, Thailand, China, USA and unknown origin sub-populations. Interestingly, there is a single alteration in the amino acid found in all individuals of the Mainland populations at the 140th site (valine to isoleucine).

Based on the geography, the Sumatran subpopulation has four haplotypes (H1, H2, H6 and H10), the Peninsular Malaysian subpopulation has two haplotypes (H1 and H7), the Thailand subpopulation has three haplotypes (H8, H9 and H12), the Borneo subpopulation has one haplotype (H9), the Cambodian subpopulation has six haplotypes (H4, H11–H13, H15–H16), the China subpopulation has one haplotype (H14), the USA subpopulation has two haplotypes (H12 and H17) and unknown samples origin has two haplotypes (H3, H5 and H14). Haplotype H12 is the haplotype with the most members shared by 12 individuals from three sub-populations. Based on the grouping in the haplotype network, two Haplogroups were formed, namely Haplogroup I and II. Haplogroup I consists of subpopulation members in the Sundaland group and Haplogroup II consists of subpopulation members in the Mainland group. Haplogroup I consists of Haplotype 1–11 (H1–H11). Among members of haplogroup I, there are

Table 3: Haplotype diversity and nucleotide diversity of *H. malayanus*

Population	n	Hn	Hd	π
Peninsular Malaysia	3	2	0.667	0.00104
Sumatra	8	4	0.821	0.00513
Borneo	1	1	0.000	0.00000
Thailand	4	3	0.833	0.01016
Cambodia	17	6	0.691	0.00391
China	2	1	0.000	0.00000
USA	2	2	1.000	0.00781

n: Number samples, Hn: Number haplotype, Hd: Haplotype diversity and π : Nucleotide diversity

19 mutated nucleotide bases, which form 11 haplotypes. The dominant haplotype is Haplotype H1 (central haplotype) as the reference sequence for *H. malayanus*. Five individuals share haplotype H1 consisting of *H. malayanus* Peninsular Malaysia (2 individuals) and *H. malayanus* Central Sumatra (3 individuals). Haplotypes H2, H6 and H10 belong to the Sumatran GenBank individuals. The Sumatran GenBank individuals differ from Central Sumatra individuals by one base (H2), four bases (H6) and five bases (H10) (Fig. 1). The *H. malayanus* Borneo shares the same haplotype (H9) with *H. malayanus* Thailand OQ564463.1. Besides, in Haplogroup I, there are also two *H. malayanus* Cambodia (OQ564469.1 and OQ564480.1) with two haplotypes (H4 and H11) and *H. malayanus* Thailand OQ564468.1 which has haplotype H8.

Haplogroup II consists of Haplotype 12-17 (H12-H17). Among members of Haplogroup II, 13 mutated nucleotide bases form 6 haplotype variations (Fig. 1). Haplotype H12 is the haplotype with the most individual members. Haplotype H12 is shared by 12 individuals from three subpopulations (Cambodia, Thailand and USA). The Cambodian sub-population has the most haplotype variations among the other sub-populations, with six haplotypes. The haplotype and nucleotide diversity value of *H. malayanus* in each subpopulation were shown in Table 3. The Haplotype diversity values in seven *H. malayanus* sub-populations range from 0 to 1. The existence of subpopulations with a value of 0 indicates that individuals share the same haplotype. The USA subpopulation has the highest haplotype diversity value of 1 because it has two individuals with two different haplotypes. Each *H. malayanus* subpopulation has a relatively high Haplotype diversity (above 0.6), except for the China and Borneo subpopulations. The Borneo subpopulation only has one individual and the China subpopulation has two individuals sharing the same haplotype. Nucleotide diversity values in seven *H. malayanus* sub-populations range from 0.000 to 0.010. Thailand's subpopulation has the highest nucleotide diversity among other subpopulations. Overall, *H. malayanus* has high haplotype diversity and low nucleotide diversity.

Phylogenetic analysis: The phylogenetic relationship of the bears' group is shown using the maximum likelihood method in the IQ Tree with 5000 bootstraps (Fig. 2). The best analysis model used is TPM2u+F+I+G4 based on BIC. The phylogenetic tree shows the relationship between Ursidae members divided into four main clusters. The spectacled bear (*Tremarctos ornatus*) formed the basal branch for the family Ursidae in cluster IV, followed by the giant panda (*Ailuropoda melanoleuca*). In cluster III, the sloth bear (*Melursus ursinus*) is the basal branch of the subfamily Ursinae. Brown and polar bears are grouped in Cluster II. Brown bears and polar bears are present in the same cluster with the low sequence divergences (3.0-3.1%) as different species. Asian black bears, american black bears and sun bears are grouped in cluster I, with american black bears as the sister taxa of the sun bear. Among all bear species, they have sequence divergence of 3.0-29.1%. Sun bear (*H. malayanus*) is present in cluster I with the three sub-clusters. Subpopulations in the Mainland group occupy subcluster 1. Subpopulations in the Sundaland group occupy subcluster 2 (Peninsula lineage) and subcluster 3 (Sabah lineage). The Mainland group and Sundaland group separated into different subclusters with sequence divergences of 1.0-2.3%. Subcluster 1 (Mainland group) comprises almost all individuals from the Cambodian subpopulation (16 individuals), China, the USA and two Thailand individuals. Members of subcluster 1 have sequence divergence of 0.0-0.8% between them.

Peninsular lineage is the regional name for Peninsular Malaysia as sequence references of the *H. malayanus*. Thus, sequences with almost similar variations with Peninsular Malaysia are grouped into the Peninsular lineage. In the phylogenetic tree, subcluster 2 (Peninsular lineage) consists of *H. malayanus* Peninsular Malaysia and *H. malayanus* Sumatra. However, *H. malayanus* from an unknown region and one Cambodian individual are also grouped in this subcluster due to having similar variations. Members of subcluster 2 have sequence divergence of 0.0-1.1% between them. Sabah is the region name for Borneo (Sabah, Sarawak, Kalimantan and Brunei). Sequences with almost similar variations with Borneo are grouped into the Sabah lineage. Subcluster 3

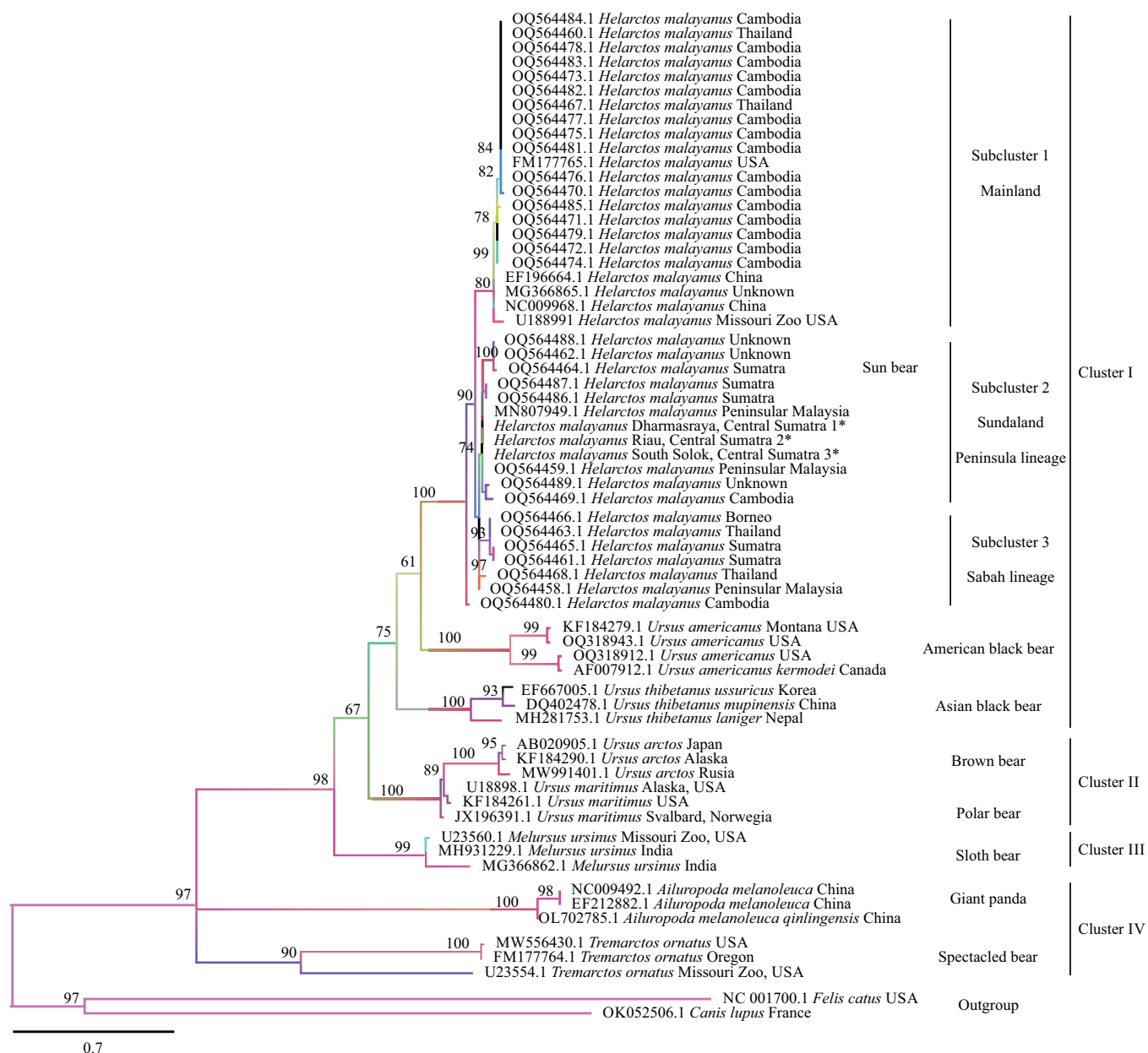


Fig. 2: Phylogenetic tree based on the *cyt b* gene (IQ Tree) of *H. malayanus* using 5000 bootstraps

(Sabah lineage) consists of *H. malayanus* Borneo (Kalimantan) and several individuals from different subpopulations (Peninsular Malaysia, Sumatra, Thailand and Cambodia) due to having similar variations. Members of subcluster 3 have sequence divergence of 0.0-1.3% between them. Subclusters 2 and 3 separated in the Sundaland group with sequence divergences of 0.5-1.4%.

DISCUSSION

Analysis using Cyt b gene data successfully represented the phylogenetic relationships of eight bear

species. Bears are one of the animals with many species that went extinct around 33.9-2.5 million years ago. In this study, phylogenetic reconstruction supports the placement of the eight extant bear species in the Ursidae family, with the spectacled bear (*T. ornatus*) as the earliest species, followed by the giant panda (*A. melanoleuca*), which is both in the same cluster (cluster IV). The previous study using complete genome mtDNA⁴¹ reported *A. melanoleuca* as the most basal member of extant bear species and present in the different clusters with *T. ornatus*. Another study using nuclear genes gave three hypotheses: *A. melanoleuca* may be the earliest species to diverge from other Ursidae or maybe *T. ornatus* as

the earliest species or *A. melanoleuca* and *T. ornatus* as a common ancestor⁴². The different positions of the giant panda and spectacled bear in the tree in the previous studies and this study could occur due to differences in evolutionary time from other genes and the numbers of genes in the analysis. Nevertheless, the results still represent the two species as the earliest and sister group. Hu *et al.*⁴³ also reported that *T. ornatus* has the closest relationship with *A. melanoleuca* among all extant bear species.

Clusters I, II and III are occupied by six species of the Ursinae subfamily. In the phylogenetic tree, the sloth bear (*M. ursinus*) is the ancestor to all bears within the Ursinae subfamily. Yu *et al.*⁴⁴ also reported *M. ursinus* as the ancestor of bear species in the Ursinae subfamily. *Melursus ursinus* is a modern bear that survives during radiation events (5.3 Mya). According to Yu *et al.*⁴¹ the sloth bear (*M. ursinus*) is a sister taxon for five species Ursinae subfamily. Lai *et al.*³³ also showed *M. ursinus* on the basal branch for the Ursinae subfamily supported by high bootstrap values. Members of the Ursinae show a monophyletic relationship with high bootstrap support. Monophyletic relationship in the Ursinae also has been reported by previous studies^{45,46} with sloth bears as the ancestor of Ursinae.

Interestingly, cluster II is shared by the brown bear (*Ursus arctos*) and the polar bear (*Ursus maritimus*). The position of the brown bear and polar bear as closely related species was also reported in previous studies^{41,45,47,48}. The relationship between brown bears and polar bears is still poorly explained. Several studies explain that this can occur due to natural mating between brown and polar bears, results hybrid offspring⁴⁸. The genome-scale studies⁴⁵ show that 8.8% of the brown bears genome originated from polar bears during the ancient hybridization. According to Liu *et al.*⁴⁹, the polar bear is the most recently formed species and descendant of the brown bear population isolated in northern latitudes by glacial events 400,000 years ago. Further study of brown and polar bears from an ABC island (type locality) will interpret the actual speciation event of polar bears more accurately⁴⁵.

Ursus americanus (american black bear), *Ursus thibetanus* (asian black bear) and *H. malayanus* (sun bear) are in the same cluster in cluster I, supported with high bootstrap value. The close relationship between *U. thibetanus* and *U. americanus* was also reported in other studies using mtDNA^{34,45,48}. Kumar *et al.*⁴⁸ stated that despite the american black bear and asian bear species being geographically separated, the fossil record, morphology and mtDNA data show their closed relationships.

Phylogenetic tree reconstruction shows *H. malayanus* in cluster I has a monophyletic relationship supported by high bootstrap value. Based on the pattern of nucleotide base variation, haplotype network and position on the phylogenetic tree, *H. malayanus* is separated into Mainland and Sundaland groups. *H. malayanus* from Sundaland and Mainland groups have specific nucleotide base variations that differentiate both groups. Certain nucleotide bases are only found in the Sundaland group and vice versa. The previous studies^{33,35} that became this study's reference supported the separation between *H. malayanus* from Sundaland and Mainland. Kunde *et al.*³⁵ revealed two lineages of *H. malayanus* consisting of lineage from mainland Indochina (China, Cambodia, Thailand) known as the "Mainland clade" and lineage from the Sundaic region (Peninsular Malaysia, Sumatra, Borneo but also Thailand) known as the "Sundaland clade".

Woodruff and Turner⁵⁰ estimated the frequent fluctuation in sea levels in Sundaland during the late Pleistocene caused discontinuity between Indochina and the Sundaland region. Lai *et al.*³³ also hypothesized that *H. malayanus* spread southwards from Indochina in the mid-Pleistocene during glacial periods when low sea levels and the Sunda Shelf were the adjacent landmass. However, during the interglacial period, sea levels rose and covered most of the Sunda Shelf area. The rising sea levels affected the separation of Sundaland (Peninsular Malaysia, Malaysia states, Borneo and Sumatra) and the Mainland. Although this study and the previous studies³³⁻³⁵ do not represent all subpopulations of *H. malayanus* due to the absence of available data, overall, the separation of *H. malayanus* into different subpopulations as assumed by Scotson³¹. Scotson³¹ stated the *H. malayanus* range is broken up naturally by the ocean into three main populations (Mainland Southeast Asia, Sumatra and Borneo). However, habitat fragmentation, human influence and road construction become potential barriers to connectivity between subpopulations and divide *H. malayanus* distribution into seven subpopulations.

The separation between *H. malayanus* Sundaland and Mainland into different subclusters is supported by the higher genetic distance between them than the genetic distance for individuals within the subpopulations of each group. Therefore, the geographic isolation between subpopulations in the Sundaland and Mainland groups causes the absence of genetic mixing/gene flow, which results in high genetic distance between these groups. Interestingly, the Sundaland group is divided into two subclusters: The Peninsular lineage (subcluster 2) and the Sabah lineage (subcluster 3). There are

several explanations regarding the separation history of *H. malayanus* in the Sundaland and Mainland Regions. According to Louys⁵¹, *H. malayanus* fossils in Indochina (Mainland) were found during the Middle Pleistocene, while the presence of *H. malayanus* in the Sundaland is dated to the late Pleistocene^{52,53}. Lai *et al.*³⁴ suggest a possibility of historical separation between *H. malayanus* in the Sundaic Region (Peninsular Malaysia, Sumatra and Borneo) and other regions in the Southeast Asian Mainland.

The samples of *H. malayanus* in Central Sumatra in this study are present in subcluster 2 (Peninsular lineage) together with other subpopulations of Peninsular Malaysia, other Sumatran individuals, several individuals of unknown sample origin and one Cambodian individual. The genetic data that resulted in this study is the first genetic data from Sumatran *H. malayanus* as a type locality collected directly. Kunde *et al.*³⁵ reported that, data on Sumatran *H. malayanus*, but samples were obtained from several historical museums in other countries. The three individuals of *H. malayanus* from Central Sumatra have a genetic distance of 0.0%, indicating no genetic differences. When compared with other *H. malayanus* individuals, *H. malayanus* from Central Sumatra has genetic similarities (100%) to Malaysian *H. malayanus*³³ and Malaysian *H. malayanus*³⁴ GenBanks. Meanwhile, *H. malayanus* in Central Sumatra has a sequence divergence of 0.3-0.7% with *H. malayanus* Sumatra GenBank³⁵. In Subcluster 3 (Sabah lineage), which is the descendant of the Borneo subpopulation, there are also several individuals from the Peninsular Malaysia, Sumatra, Thailand and Cambodia subpopulations. Interestingly, one Thailand individual shares the same haplotype with *H. malayanus* Borneo (Sabah lineage). These results show some individuals from the Mainland subpopulations (Cambodia and Thailand) have higher genetic similarities with individuals from the Sundaland subpopulations, even though Cambodia and Thailand are geographically included in the Mainland Region.

Kunde *et al.*³⁵ also reported *H. malayanus* Borneo sharing the same haplotype with one Thailand individual and the presence of overlapping subpopulations in the Sabah lineage. The other study³³ using several mtDNA genes also showed *H. malayanus* Thailand has two lineages from Mainland and Sundaland. The haplotype diversity and tree topology suggest the presence of Thailand lineage in the Sundaland group, which appears to have recently evolved from Malaysian ancestors. According to Lai *et al.*³³, the hypothesis for explaining the presence of two lineages in Thailand is that if the loss of Mainland lineage occurred before or during the colonization of the Sundaland, this indicates *H. malayanus*

Sundaland's lineage in Thailand has survived since the Middle Pleistocene. However, if the loss of mainland lineage occurred after colonization, this suggests the existence of connectivity/secondary contact of two lineages (Mainland and Sundaland) in Thailand. Migration was possible when the mainland and large islands of Sundaland were connected during low sea levels in the late Pleistocene⁵⁴. Meanwhile, *H. malayanus* Borneo, Kalimantan was not found in Peninsular lineage, possibly due to the lack of available data. However, a study using the mtDNA gene³³ with a more significant number of samples from Peninsular Malaysia and Borneo subpopulations revealed the absence of *H. malayanus* Borneo (Sabah lineage) in Peninsular lineage. Then, it generates the question of why the two lineages do not coexist in Borneo³⁵. Thus, further molecular studies (mtDNA and nuclear DNA) with an additional number of samples from other subpopulations, especially Borneo, are needed to confirm the taxonomic uncertainty regarding two subspecies of *H. malayanus*^{33-35,55}.

Overall, nucleotide base variations have led to missense mutations that cause changes in five amino acids in several *H. malayanus* individuals. Interestingly, one amino acid change is shared in all members of the Mainland group. Based on the function, the Cyt b gene is a protein-coding gene in mtDNA for respiration. A missense mutation causes changes in structure and malfunction protein. However, not all missense mutations cause changes in structure and function. If an amino acid replaces an amino acid with similar chemical characteristics, the protein can still function normally, called a conservative mutation. However, if an amino acid replaces an amino acid with a different function and characteristics, it can deactivate the protein's function, called a non-conservative mutation.

The amino acids that change in *H. malayanus* are replaced by amino acids with almost the same function and chemical characteristics. Thus, the protein can still function normally, which is included in the conservative mutation. In this study, some examples are the amino acid changed from threonine to alanine, which proteins play a role in the body's immunity and valine to isoleucine, which plays a role in growth and muscle regeneration. Thus, the changes in amino acids that occur do not change the structure and protein function. Several factors such as ecology, food type and food habits, environmental pressure and other factors that differ among subpopulations in the Mainland and Sundaland groups are estimated to play a role in genetic variations between groups.

CONCLUSION

All individuals of *H. malayanus* in Central Sumatra have the same haplotype as the Peninsular Malaysia sequence. The sun bear (*H. malayanus*) has a monophyletic relationship with other bear species. The study results support sun bear (*H. malayanus*) divided into two different lineages: Mainland (subcluster 1) and Sundaland (subcluster 2 and 3). The geographic isolation causes the absence of gene flow, which results in high genetic distance between Sundaland and Mainland lineages.

SIGNIFICANCE STATEMENT

The geographical isolation between subpopulations in sun bears (*H. malayanus*) allegedly impacts morphological and genetic variations. Although Sumatra is a type locality of the sun bear, the biological data available for the Sumatran population is limited, especially morphological and genetic data. Therefore, providing data and information on the Sumatran sun bear is becoming essential. This study aimed to determine the phylogenetic relationship and haplotype diversity of sun bears in Sumatra and other Southeast Asian Regions. These data will be helpful as a reference to determine the appropriate conservation strategies for the Sumatran sun bear.

ACKNOWLEDGMENTS

Andalas University supported the research under the RPB scheme (No.T/10/UN.16.19/PT.01.03/IS-RPB/2023). The authors sincerely thank the Head of the Biology Department of Andalas University for the Laboratory work permit. We also would like to thank our team of geneticists who helped us with sample collection and laboratory work in the Genetic and Biomolecular Laboratory, Faculty of Mathematics and Sciences Andalas University, Padang, Indonesia.

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