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GENETIC ANALYSIS OF SUMATRAN ELEPHANTS IN SEBLAT NATURAL ECOTOURISM PARK BASED ON PARTIAL OF MITOCHONDRIAL CYTOCHROME B GENE

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ABSTRACT

Sumatran elephant (*Elephas maximus sumatranus* Temminck, 1847) is one of the Asian elephant sub-species distributed in Sumatera Island and classified as endangered species due to hunting and high habitat fragmentation. This study aimed to analyze the genetic relationship of Sumatran elephants based on the mitochondrial cytochrome b gene (*Cyt b*). Blood samples were collected from 11 elephants in Seblat Natural Ecotourism Park. Based on mt-DNA Cytochrome b gene analysis, low genetic diversity was found in the Sumatran elephant population, indicated by 99-100% sequence similarity among elephant samples. Phylogenetic analysis showed that all elephants were in the same clade and has a close relationship with Borneo elephants. Additionally, the Median-joining network illustrated only two haplotypes in the Sumatran elephant population. The low genetic diversity of the Sumatran elephant indicates that a strategic breeding program should be seriously taken into account to prevent the Sumatran elephant from extinction.

Key words: Cytochrome b, genetic diversity, mitochondrial DNA, Sumatran elephant

INTRODUCTION

Sumatran elephant (*Elephas maximus sumatranus* Temminck, 1847) is one of the Asian elephant sub-species distributed across the Sumatran mainland. The Sumatran elephant has been classified as a critically endangered species under the International Union for Conservation of Nature and Natural Resources (IUCN) Red List (Gopala *et al*., 2011). World Wide Fund for Nature (WWF) Indonesia, estimated that the Sumatran elephant population was about 2,400-2,800, and the population continuously reduced due to the lack of habitat and hunting activity (WWF 2008).

Fragmentation and habitat loss are primary factors of a globally declined population (Gopala *et al*., 2011; Hermes *et al*., 2013). Fragmentation and isolation of populations lead to demographic problems and shrinking genetic diversity due to limited gene flow between elephant populations. Limited gene flow may cause genetic drift, inbreeding, loss of allelic expression, and decrease disease resistance (Allendorf *et al*., 2012). Thus, it is necessary to preserve Sumatran elephants and maintain their genetic diversity as part of conservation genetic strategies. With genetic analysis, it is possible to study status, distribution, kinship, ancestry, and genetic inheritance. Genetic analysis can be done using different techniques, one of which is based on mitochondrial DNA (mtDNA) genes for determining diversity and kinship. Fleischer *et al*. (2001) reported that mtDNA variation suggests that the Sumatran subspecies is monophyletic and consequently could be defined as an evolutionarily significant unit (ESU). A previous study reported by Sulandari and Zein (2012) described that low haplotype and nucleotide diversities were found in Sumatran elephant populations based on the D-loop region of mtDNA. The low genetic diversity of the Sumatran elephant originating from the South Sumatera area is also reported by Wibowo *et al*. (2021) using mtDNA Cytochrome Oxidase subunit II (*COX2*). Moreover, Virnarenata *et al*. (2021) reported that a close genetic relationship was found in the Sumatran elephant conserved in Way Kambas National Park based on mtDNA Cytochrome Oxidase subunit I (*COI*). Previous studies have found low levels of genetic diversity in Borneo elephants too. Fernando *et al*. (2003) compared mitochondrial

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DNA (mtDNA) and microsatellite diversity for Asian elephant populations and found a single mitochondrial haplotype in Bornean elephant samples analyzed from Sabah.

Another Sumatran elephant habitat in Indonesia is Natural Ecotourism Park (TWA), Seblat, North Bengkulu, and currently, there are 11 domesticated Sumatran elephants there. Meanwhile, genetic analysis using mtDNA Cytochrome b (*Cyt b*) of those captive elephants has never been conducted and reported in Indonesia. Therefore, we conducted a genetic analysis of Sumatran elephants in Natural Ecotourism Park (TWA), Seblat, North Bengkulu for determining their genetic diversity based on the mtDNA *Cyt b* gene.

MATERIALS AND METHODS

Samples collection and preparation

Blood samples were collected for a month on August 2018 from a total of 11 individuals of Sumatran elephants in Natural Ecotourism Park (TWA), Seblat, North Bengkulu (Table 1). Blood samples were put into a 5 mL tube containing EDTA using 21G 2 mL syringes from the elephant's ears. They were kept at -21 °C until further analysis.

Extraction of genomic DNA

A total of 100 µL of blood was used for DNA extraction following Geneaid Blood and Tissue Mini Kit (GS100, GS300) protocol. The DNA purity was checked using a UV-Vis spectrophotometer (Thermo Scientific Evolution 200 series, United State) at a wavelength of 260 nm and 280 nm.

DNA amplification and visualization

A total length of 630 bp mtDNA *Cyt b* gene fragment was amplified using MDL 3 [5'-CCCACAATTAATGGGCCCGGAGCG-3' and MDL 5 [5'-TTACATGAATTGGCAGCCAACCAG-3' primers (Fernando *et al*., 2000). The PCR reaction started with pre-denaturation at 94 °C for 4 min, and it was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 ºC for 45 s, extension at 72 °C for a min, and final extension at 72 °C for 7 min. The PCR product was pipetted into 1% agarose gel and run for 30 min at 100 Volt. Then, the gel was stained with ethidium bromide for 15 min, followed by washing with ddH_2O for 1 min. Visualization of DNA was performed under UV light using the Gel Documentation System (Axygen). The PCR product was sent to PT. Genetika Sains Indonesia and forwarded to first Best Malaysia for sequencing

Data analysis

Phylogenetic analysis was performed by using Molecular Evolutionary Genetics Analysis (MEGA) X software (Kumar *et al*., 2018). A phylogenetic tree was constructed using the Neighbor-Joining tree method with 1000x bootstrap replication. Mitochondrial DNA *Cyt b* sequences obtained in this study, the NCBI website, and out-group sequences (Table 2) were used to construct the phylogenetic tree. Furthermore, the *Cyt b* gene sequences we got were compared with the other data available on the NCBI website using the Basic Local Alignment Search Tool Nucleotide (BlastN). In the final analysis, the Network v.5.0.0.1 software was used to determine the haplotype network among the population used in this study (Kumar *et al*., 2018).

Table 1. Profile and DNA purity of 11 captive Sumatran elephants (*Elephas maximus sumatranus* Temminck, 1847) in Natural Ecotourism Park (TWA) in Seblat, North Bengkulu

Elephant Identity	Gender	Origin	Wavelength (nm)		Concentration (ng/µL)
			260	280	
Mega (MG)	Female	Ipuh, Muko-Muko			
Anggarini (AG)	Female	Ipuh, Muko-Muko	0.074	0.058	551.30
Desi (DS)	Female	Seblat, North Bengkulu	0.065	0.054	464.75
Sari (SR)	Female	Ipuh, Muko-Muko	0.066	0.053	491.70
Devi (DV)	Female	Ipuh, Muko-Muko	0.080	0.063	596.00
FT	Female	Ipuh, Muko-Muko	0.056	0.049	417.20
Darmi (DR)	Female	Ipuh, Muko-Muko	0.064	0.052	476.80
Bona (BN)	Female	PT. Alno Group, Air Rami, North Bengkulu	0.072	0.057	536.40
Robi (RB)	Male	Ipuh, Muko-Muko	0.077	0.062	573.65
Nelson (NL)	Male	Seblat, North Bengkulu	0.070	0.056	521.50
Ucok (UC)	Male	Seblat, North Bengkulu	0.071	0.058	528.95
			0.069	0.056	514.05

Table 2. The accession number of mtDNA Cyt b sequences obtained in this study and out-group sequences based on NCBI

mtDNA Cyt-b sequences obtained in this study	Accession Number (NCBI)
	AY245825.2
	AY245812.2
	AY245825.2
	FJ979436.1
	AY245825.2
	FJ979437.1
	AY245825.2
	AY245803.2
	AY245825.2
	AY245811.2
	AY245825.2
	KJ187801.1
	KJ187789.1
	AY245812.2
	AY245816.2
	AY245813.2
	AY245824.2
	AY245814.2
	FJ979514.1
	HQ113851.1
	AY245810.2
	AY245806.2
mtDNA Cyt-b sequences of outgroup	AF132520.1
	EU116015.1
	AF132526.1
	MN14748.1
	FJ753556.1

RESULTS

The DNA quantification

The DNA quantification results showed that extracted genomic DNA of 11 captive elephants was in high concentration. It ranged from 491.7 to 596 ng/µL (Table 1). Devi (DV) was the highest DNA concentration (596 ng/µL), while FT was the lowest DNA concentration (417 ng/µL). The total concentration of genomic DNA to amplify more than 500 base pairs of DNA fragments was recommended as 40 ng/µL (First Base DNA Sequencing Service 2000). Therefore, the DNA concentration extracted from elephants can be used for sequencing analysis. The result of DNA amplification showed that the amplicon size was 630 base pairs (Figure 1).

Analysis of *Cyt b* gene sequence in Sumatran elephant

Sequences of the Sumatran elephant have been registered and can be accessed at National Center for Biotechnology Information (NCBI) website. Basic local alignment search tool (BLAST) analysis showed that all elephants were closely related to *Elephas maximus*. The similarity value between the elephant *Cyt b* gene sequence with a published sequence on the NCBI website ranged from 98.49 to 100% (Table 3). A high similarity percentage (99 to 100%) indicated that elephants were identified as the same species as *Elephas maximus*. Based on the alignment of samples with two Asian elephant subspecies, *E. maximus maximus,* and *E. maximus indicus*, all samples are closely related to *E. maximus borneensis.*

All sequences obtained in this study were then aligned to find out polymorphisms within the *Cyt b* gene fragment. A total of 25 polymorphism sites were found in the Sumatran elephant population compared to published sequences. All Sumatran elephants were genetically similar due to only one polymorphism found within this population at the position of 001, which has the same Timine between Darmi (DR) and Robi (RB) (Table 3).

Phylogenetic tree and network analysis of Sumatran elephant

The phylogenetic tree was constructed for 11 captive Sumatran elephants from TWA, Seblat, and North Bengkulu with comparative elephant genes from the gene bank (Figure 2). In this figure, there were two main clusters. Cluster I grouped 34 elephants that segregated two individuals, HQ113851.1 for species with LaoPDR-E haplotype and AJ303056.1 for having

mitochondrial partial *Cyt b* gene-Israel, while Cluster II was formed based on 495th nucleotide (counted from the first base of PCR product) consisting of Adenine (A) with bootstrap value was 54 (Table 4). Figure 2 also showed that 11 captive Sumatran elephants have a close relationship with Asian elephants as shown by the 100% of similarity value. A high similarity value (100%) indicates a close relationship among 11 Sumatran elephants in TWA, Seblat.

Median Joining Network (MJ-Network) analysis was conducted to get haplotype distribution and haplogroup of Sumatran elephants and published sequences on the NCBI website. A total of 18 haplotypes with 16 parsimony variable sites and 10 singleton variable sites have been identified in this study (Table 4). Sumatran elephants were grouped into two haplotypes, i.e. haplotypes 1 and 2. Nine Sumatran elephant samples were grouped into haplotype 1 which is not shared with other elephants. Moreover, two Sumatran elephants were grouped into haplotype 2 which is shared with the Borneo elephant (AY245825.2) (Figure 3). These findings indicated that Sumatran elephants have a close relationship with the Borneo elephant.

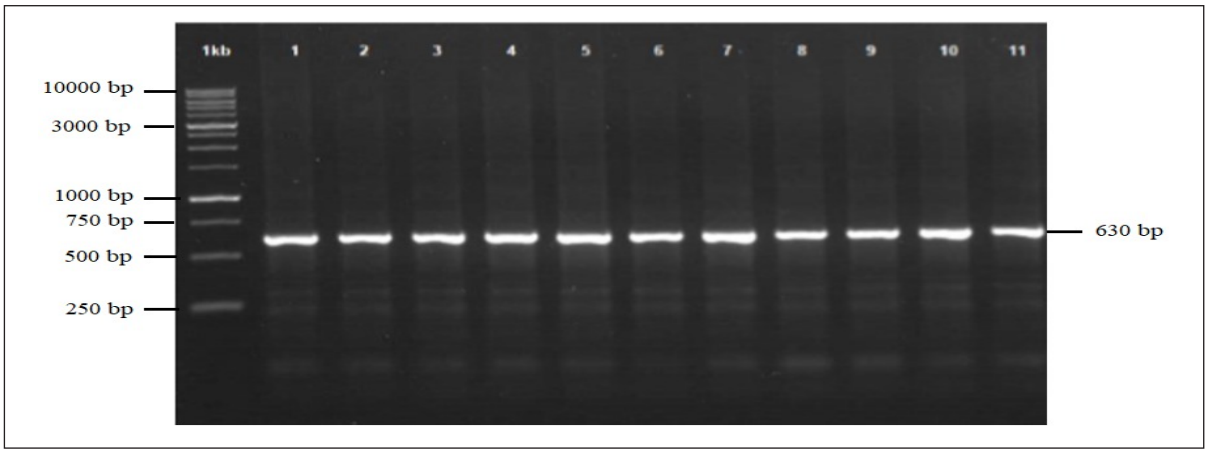

Fig. 1. The PCR product of mtDNA Cyt b gene in Sumatran elephant population. 1kb is a 1 kb marker ladder; 1 to 11 are Sumatran elephant samples.

Table 3. Alignment of partial Cyt b gene of Sumatran elephants *(Elephas maximus sumatranus* Temminck, 1847) compared with Asian elephants *(Elephas maximus*) at Genebank

Sample name	Species affiliation	Similarity (%)	Origin species	Accession Number
Mega (MG)	*Elephas maximus* haplotype BT	100.00	Borneo	AY245825.2
	Elephas maximus haplotype BU	99.83	Borneo	AY245812.2
Anggraini (AG)	*Elephas maximus* haplotype BT	100.00	Borneo	AY245825.2
	Elephas maximus isolate 24	99.15	North America	FJ979436.1
Desi (DS)	*Elephas maximus* haplotype BT	100.00	Borneo	AY245825.2
	Elephas maximus isolate 26	99.15	North America	FJ979437.1
Sari (SR)	*Elephas maximus* haplotype BT	100.00	Borneo	AY245825.2
	Elephas maximus haplotype BH	99.15	Borneo	AY245803.2
Devi (DV)	*Elephas maximus* haplotype BT	100.00	Borneo	AY245825.2
	Elephas maximus haplotype BR	99.15	Borneo	AY245811.2
Fatma (FT)	*Elephas maximus* haplotype BT	100.00	Borneo	AY245825.2
	Elephas maximus isolate EmTHb13	99.15	Thailand	KJ187801.1
Darmi (DR)	*Elephas maximus* isolate EmTHb01	99.15	Thailand	KJ187789.1
	Elephas maximus haplotype BU	99.83	Borneo	AY245812.2
Bona (BN)	*Elephas maximus* haplotype BQ	99.15	Borneo	AY245816.2
	Elephas maximus haplotype BP	99.15	Borneo	AY245813.2
Robi (RB)	*Elephas maximus* haplotype BG	98.98	Borneo	AY245824.2
	Elephas maximus haplotype BV	98.98	Borneo	AY245814.2
Nelson (NL)	*Elephas maximus* isolate 199	98.98	North America	FJ979514.1
	Elephas maximus haplotype LaoPDR-E	98.49	Nakai Plateau, Laos	HQ113851.1
Ucok (UC)	*Elephas maximus* haplotype BK	98.98	Borneo	AY245810.2
	Elephas maximus haplotype BM	98.98	Borneo	AY245806.2

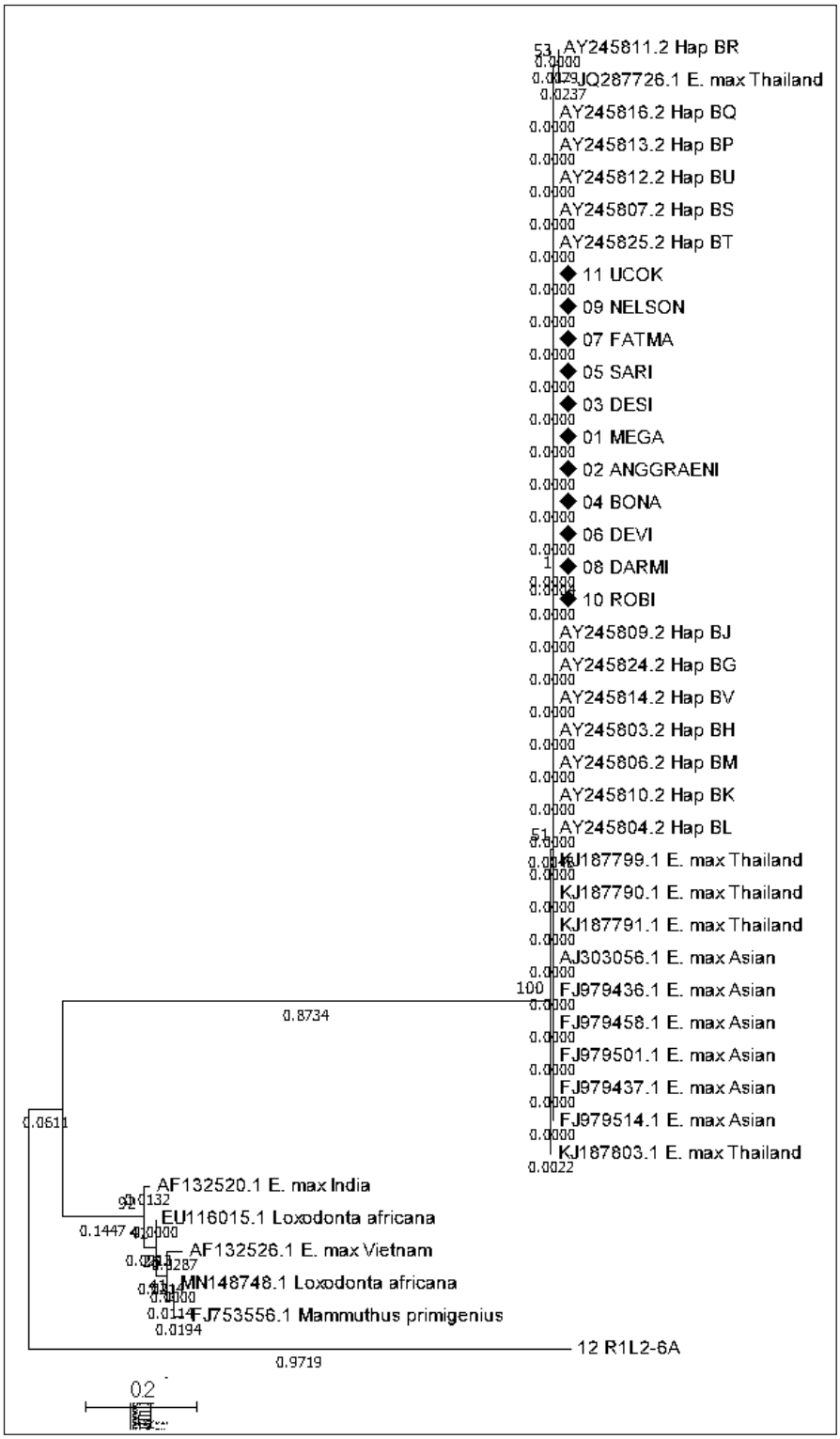

Fig. 2. Phylogenetic tree of 11 captive Sumatran elephants (*Elephas maximus sumatranus* Temminck, 1847) and outgroup. Construction is based on the neighbor-joining tree method with a 1000x bootstrap replication value.

Table 4. Nucleotide site of partial *Cyt b* gene Sumatran elephants (*Elephas maximus sumatranus* Temminck, 1847) compared with Asian elephants (*Elephas maximus*) at Genebank

	Position																								
Elephant	001	005	048	128	141	204	250	373	380	382	392	393	403	404	418	420	430	442	445	472	490	495	526	553	557
Female 1 (MG)	A	C	T	A	G	G	C	T	A	T	A	C	T	G	T	T	A	T	A	A	A	G	T	C	A
Female 2 (AG)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Female 3 (DS)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Female 4 (SR)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Female 5 (DV)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Female 6 (FT)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Female 7 (DR)	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Female 8 (BN)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Male 1 (RB)	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Male 2 (NL)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Male 3 (UC)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AY245825.2	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AY245807.2	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AY245812.2	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
FJ979436.1	T	T	.	G	.	.	.	.	.	.	G	.	.	.	.	C	.	C	.	.	.	.	.	.	.
FJ979458.1	T	.	.	G	.	.	.	.	.	C	.	.	C	.	.	.	G	C	.	.	.	.	.	.	.
AY245813.2	T	.	C	G	.	.	T	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.
AY245816.2	T	.	.	G	.	.	.	.	.	C	.	.	C	.	.	.	G	C	.	.	.	.	.	.	.
KJ187789.1	T	.	.	G	.	.	.	.	.	C	.	.	C	.	.	.	G	C	.	.	.	.	.	.	.
FJ979501.1	T	T	.	G	.	.	.	.	.	.	G	.	.	.	.	C	.	C	.	G	.	.	.	.	.
AJ303056.1	T	.	.	G	A	.	.	.	G	.	G	.	.	.	.	C	.	C	.	.	.	A	.	T	.
FJ979437.1	T	.	C	G	.	.	T	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.
AY245809.2	T	T	.	G	.	.	.	.	.	.	G	.	.	.	C	.	.	C	.	.	.	.	.	.	.
AY245811.2	T	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.
KJ187801.1	T	T	.	G	.	A	.	.	.	.	G	.	.	.	.	C	.	C	.	.	.	.	.	.	.
KJ187791.1	T	.	C	G	.	.	T	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.
HQ113851.1	T	.	.	.	.	.	.	C	G	.	.	.	.	.	C	C	.	C	.	.	G	A	C	T	.
FJ979514.1	T	T	.	G	.	.	.	.	.	.	G	.	.	.	.	C	.	C	.	.	.	.	.	T	.
AY245824.2	T	T	.	G	.	.	.	.	.	.	G	.	.	.	.	C	.	C	.	G	.	.	.	.	.
AY245814.2	T	.	.	G	.	.	.	.	.	C	.	.	C	.	.	.	G	C	.	.	.	.	.	.	C
AY245803.2	T	T	.	G	.	.	.	.	.	.	G	.	.	.	.	C	.	C	.	.	.	.	.	.	.
AY245806.2	T	T	.	G	.	.	.	.	.	.	G	.	.	.	.	C	.	C	.	.	.	A	.	.	.
AY245810.2	T	T	.	G	.	.	.	.	.	.	G	.	.	A	C	.	.	C	.	.	.	.	.	.	.
KJ187790.1	T	.	.	G	.	.	.	.	.	C	.	T	C	.	.	.	G	C	.	.	.	.	.	.	.
AY245804.2	T	T	.	G	.	.	.	.	.	.	G	.	.	.	.	C	.	C	.	.	.	.	.	T	.
KJ187803.1	T	T	.	G	.	.	.	.	.	.	G	.	.	.	.	C	.	C	.	.	.	.	.	.	.

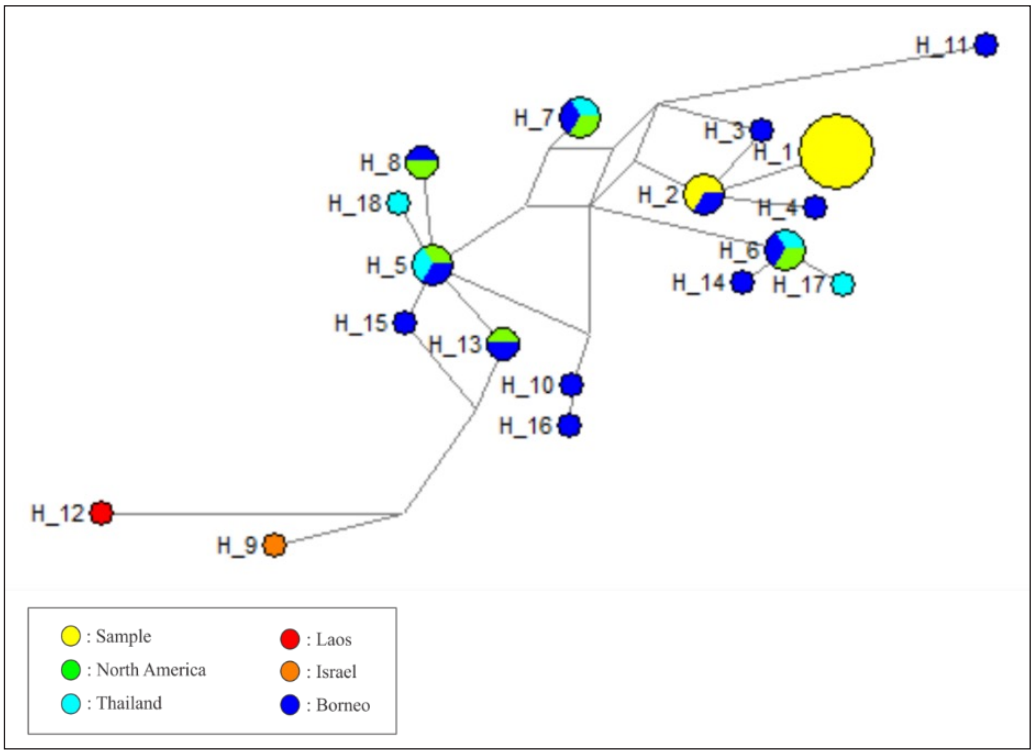

Fig. 3. Network analysis of mtDNA Cyt b gene sequences. H is a haplotype. The yellow region indicates the sample (Sumatran elephants).

DISCUSSION

High similarity sequences among Sumatran elephants were found in this population which indicated their genetic variation was low. It might be due to founder events, bottlenecks, genetic drift, inbreeding, and small population size for a long time (Frankham *et al*. 2010). A previous study reported by Sulandari & Zein (2012) stated that there were 21 Sumatran elephants in Seblat captivity. Currently, we found only 11 Sumatran elephants there. This result indicated that Sumatran elephants in Seblat National Park were critically endangered. A sustainable breeding program is extremely needed to improve their population in Seblat. This high similarity also suggested that mtDNA diversity, especially in the Cytochrome b gene region, is very low in the Seblat. This result is in agreement with previous studies using other mtDNA fragments, such as D-loop, *COXII*, and *COI* (Sulandari & Zein, 2012; Wibowo *et al*., 2021; Virnarenata *et al*., 2021).

The low genetic diversity of Sumatran Island may also increase higher reproductive failure because removal of heterozygous alleles. Habel and Zachos (2013) stated that low genetic diversity might be due to inbreeding, which causes the existence of homozygous individuals, mutation damage of recessive alleles, bottlenecks effect, and gene flow imbalance. Previous studies that showed the Sumatran elephant experienced a decline in terms of low genetic diversity by using other mtDNA targets have been reported by some researchers (Hedges *et al*., 2005; Sulandari & Zien 2012; Nur *et al*., 2019). Conservation efforts can be conducted to prevent the extinction of the Sumatran elephant (*Elephas maximus sumatranus* Temminck, 1847). The number of Sumatran elephants in Seblat is decreased by year. It can be a serious problem to maintain the elephant population. Strategic action should be designed to increase Sumatran elephant diversity and their number by introducing foreign elephants from other regions or by artificial insemination, especially in Natural Park (TWA), Seblat. The elephant breeding program should also be deeply studied to create a sustainable program. According to Heber *et al*. (2012), high heterogeneity in a population likely rise the potency of breeding, so allele diversity will increase in the population.

This study found two haplotypes of the Sumatran elephant (haplotype 1 & 2). These two haplotypes were also previously reported by Sulandari and Zein (2012) which also have low genetic diversity as in this study. This study showed that most elephant samples were grouped into haplotype 1, and only two samples were grouped into haplotype 2 which deviated from haplotype 1. Sumatran elephant has haplotypes that are different from the elephant in India (Ahlering *et al*., 2011; Elliza *et al*., 2015). However, introduction from Borneo elephant was found based on haplotype analysis in this study. This probability may be happening because animals can easily migrate during the Pleistocene glaciations period, much of the Sunda

shelf was exposed and the western Indo-Malayan archipelago formed a single landmass designated as Sundaland (MacKinnon *et al*., 1996). Borneo elephant has six haplotypes that share with other elephants from Thailand, Malaysia, and South Asia (Vidya *et al*., 2011). Because the haplotype has low genetic diversity, further studies are needed to increase or even maintain the Sumatran elephant population such as in breeding strategies between elephants.

CONCLUSION

In conclusion, the genetic diversity of Sumatran elephants in Seblat was very low based on a partial fragment of the mtDNA Cytochrome b gene. Sustainable breeding programs and conservation should be taken into account to improve the genetic diversity of Sumatran elephants in Seblat, therefore they can be saved from extinction. One of the recommended breeding strategies is a genetic exchange between captive populations and wild groups. To reach successful output in a breeding program, it should observe several alterations, such as reducing overwork, increasing social interaction, feeding them effectively, etc.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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