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# Sumatran tiger identification and phylogenetic analysis based on the CO1 gene: Molecular forensic application

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**Abstract.** *Ashrifurrahman, Simamora S, Ritonga R, Novarino W, Tjong DH, Rizaldi, Syaifullah, Roesma DI. 2022. Sumatran tiger identification and phylogenetic analysis based on the CO1 gene: molecular forensic application. Biodiversitas 23: 1788-1794.* Wild animal hunting, especially in the Sumatran tiger (*Panthera tigris sumatrae*), has been caused the population decline. Regulation and law enforcement have been implemented even though it does not affect optimal because of the trickery of poachers and illegal traders. Sometimes, the evidence of *P. t. sumatrae* derivative products, for example, bones, nails, skins, hair, and other body parts, cannot be properly identified to raise the cases. However, genetic markers, such as the CO1 gene, have successfully identified illegal trafficking samples. This study used 20 samples, consisting of seven samples (four preserved hairs, two claws, one bone) that were suspected of *P. t. sumatrae* collected from illegal wildlife trade cases in West Sumatra, Indonesia. Other thirteen samples were twelve blood and one hair of *P. t. sumatrae* samples were collected from the Dharmasraya Sumatran Tiger Rehabilitation Center (PR-HSD). All samples were isolated, Polymerase Chain Reaction (PCR), sequenced, and 999 base pairs (bp) of the CO1 gene sequences were analyzed. In addition, National Center for Biotechnology Information (NCBI) data sequences including two *P. t. sumatrae* sequences, four *P. t. altaica* sequences, one *P. t. amoyensis* sequence, one *P. t. corbetti* sequence, three *P. pardus* sequences, and one *Felis catus* sequence were collected for comparison and supporting data. The result confirmed that all samples in this study were *P. t. sumatrae*. We determined those depending on similarity value which was 99.60%-99.70% with *P. tigris* reference sequence (NC_010642.1) and 99.90%-100% with *P. t. sumatrae* (JF357969.1). Phylogenetic analysis supported species identification with average intraspecies sequence divergence was 0 to 0.4% and presented the monophyletic group. This study was the first and most recent report to use seized samples to identify *P. t. sumatrae* based on the CO1 gene in West Sumatra, Indonesia.

**Keywords:** CO1 gene, Forensic, Illegal hunting, *Panthera tigris sumatrae*

## INTRODUCTION

Tiger (*Panthera tigris* Linnaeus, 1758) morphological studies, geographic distribution, and molecular data revealed the phylogenetic ancestry of tigers in the world. There are nine subspecies of tiger which are Bengal tiger (*P. t. tigris*), Siberian tiger (*P. t. altaica*), South China tiger (*P. t. amoyensis*), Indochinese tiger (*P. t. corbetti*), Caspian tiger (*P. t. virgate*) (extinct), Malayan tiger (*P. t. jacksoni*), Bali tiger (*P. t. balica*) (extinct), Javan tiger (*P. t. sondaica*) (extinct), and Sumatran tiger (*P. t. sumatrae*) (Luo et al. 2004; Kitpipit et al. 2012; Xue et al. 2015; Liu et al. 2018). *Panthera tigris sumatrae* is one of six surviving subspecies. Currently, *P. t. sumatrae* is a priority animal protected by the Indonesia minister of environment and forestry regulation number P. 106 in 2018 (Permen LHK 2018). Furthermore, the International Union for Conservation of Nature (IUCN) Red List categorizes these subspecies as critically endangered because of this population reduction (Dinerstein et al. 2006).

Human activities have been the major factor that contributed to the *P. t. sumatrae* population oppression. The conversion of forest areas for plantation and settlement expansion resulted in habitat fragmentation and pushed the tigers into residential areas (Seidensticker et al. 1999; Wibisono and Pusparini 2010). Hunting and killing the *P. t. sumatrae* for illegal trade activity are other factors (Seidensticker et al. 1999). From 1990 to 2000, it was recorded that 619 tigers were killed in six provinces on the island of Sumatra (Tilson et al. 2007). Furthermore, in 2002 the authorities had successfully revealed and collected more than 414 kg of products from *P. t. sumatrae* parts, in 2006 over 202 kilograms from the wildlife trading case (Soehartono et al. 2007). However, it is necessary to take more effort to process and uncover the illegal trafficking case because it still exists. Sometimes, the pieces of evidence are incomplete and difficult to identify (Alacs et al. 2010; Forum Harimau Kita 2019).

Morphological and chemical approaches are the methods for various interests in animal identification (Bell 2011; Knecht 2012; Mariacher et al. 2019). Morphological identification has limitations when the sample is incomplete and degraded. Therefore, morphological methods still have limitations in the information obtained

(Alacs et al. 2010; Mariacher et al. 2019). DNA identification technique currently being the best way. DNA content provides information on species, individual sex, and geo-location of poached animals with relatively high accuracy (Dalton and Kotze 2011; Karmacharya et al. 2018). Mitochondrial DNA, especially the CO1 gene marker has been used for species identification and phylogenetic analysis (Hebert et al. 2003; Miller 2007; Bucklin et al. 2011; Ashrifurrahman et al. 2019; Khedkar et al. 2019). In addition, mitochondrial DNA is the basis for the taxonomic determination of tigers (Luo et al. 2004; Wilting et al. 2015).

In Indonesia, the illegal trafficking of *P. t. sumatrae* is not yet resolved. However, regulation and law enforcement have been implemented, although they still have problems (Gokkon 2021). Sometimes, the evidence of *P. t. sumatrae* such as bones, nails, skin, hair, flesh, and other body parts has not been identified properly to raise the cases exactly in West Sumatra. The previous study was successfully used DNA profiling in wildlife forensics for sea turtles (*Chelonia mydas*) (Pertiwi et al. 2020); African forest elephant (*Loxodonta cyclotis*), African bush elephant (*Loxodonta Africana*), Asian elephant (*Elephas maximus*) (Lee et al. 2013); Leopard (*P. pardus*) (Mondol et al. 2015); and Tiger (*P. tigris*) in Nepal (Karmacharya et al. 2018).

However, to uncover and eradicate cases of illegal trade of *P. t. sumatrae* must be intensified (Pusparini et al. 2017). This study aims to identify seven putative *P. t. sumatrae* parts seized by West Sumatra Natural Resources Conservation Agency (BKSDA). The molecular forensic approach based on the CO1 gene was used. Seized forensic samples were analyzed and combined with thirteen *P. t. sumatrae* samples from the natural habitat landscape. It can provide information regarding the identification of seized samples and the phylogenetic relationship between *P. t. sumatrae* in several locations. The results highlight that the CO1 gene successfully identified illegal trafficking samples of *P. t. sumatrae*. The report is the first study that applies a molecular forensic approach based on the CO1 gene to identify the *P. t. sumatrae* seized samples.

## MATERIALS AND METHODS

### Sample collection

The sample in this study consists of suspected *P. t. sumatrae* body parts and *P. t. sumatrae* samples. *P. t. sumatrae* suspected samples (four preserved hair, one bone, and two claws) were collected from West Sumatra Natural Resources Conservation Agency (BKSDA), Indonesia. The suspected *P. t. sumatrae* body parts were seized from illegal traffickers in Sumatra. In addition, *P. t. sumatrae* samples (twelve blood and one hair) were collected from the Dharmasraya Sumatran Tiger Rehabilitation Center (PR-HSD), blood samples stored at -20°C. The blood samples were collected from individual *P. t. sumatrae*, rehabilitated due to human conflict, and then released into the wild.

**Tabel 1.** *Panthera tigris sumatrae* data samples

| Samples | Sample Id | Sample type |
|---|---|---|
| *P. t. sumatrae 1* (suspected) | PTS1 | Preserved hair |
| *P. t. sumatrae 2* (suspected) | PTS2 | Preserved hair |
| *P. t. sumatrae 3* (suspected) | PTS3 | Preserved claw |
| *P. t. sumatrae 4* | PTS4 | Hair |
| *P. t. sumatrae 5* (suspected) | PTS5 | Preserved hair |
| *P. t. sumatrae 6* (suspected) | PTS6 | Preserved bone |
| *P. t. sumatrae 7* (suspected) | PTS7 | Preserved hair |
| *P. t. sumatrae 8* (suspected) | PTS8 | Preserved claw |
| *P. t. sumatrae 9* | PTS9 | Blood |
| *P. t. sumatrae 10* | PTS10 | Blood |
| *P. t. sumatrae 11* | PTS11 | Blood |
| *P. t. sumatrae 12* | PTS12 | Blood |
| *P. t. sumatrae 13* | PTS13 | Blood |
| *P. t. sumatrae 14* | PTS14 | Blood |
| *P. t. sumatrae 15* | PTS15 | Blood |
| *P. t. sumatrae 16* | PTS16 | Blood |
| *P. t. sumatrae 17* | PTS17 | Blood |
| *P. t. sumatrae 18* | PTS18 | Blood |
| *P. t. sumatrae 19* | PTS19 | Blood |
| *P. t. sumatrae 20* | PTS20 | Blood |

### Laboratory methods

The primers were designed with the Primer3Plus (Untergasser et al. 2007). The primers were expected to amplify about 1000 bp of CO1 gene sequences. Then, the primers were calibrated in Primer-BLAST with reference sequences from *P. tigris* (NC_010642.1) (Wei et al. 2011). Finally, DNA isolation for each sample was performed using GeneAll Exgene Genomic DNA micro. Each type of sample used a different protocol according to the kit guide.

PCR (Polymerase Chain Reaction) used the Sensoquiest Labcycler machine. Amplification process performed by PTSF 5'AGTTACTGCCCATGCCTTTG3' (forward primers), and PTSR 5' CAGGCCTAGGAAATGCTGAG 3' (reverse primers). PCR reactions used 25 µL volume with containing supermix Bioline 11 µL, Nuclease free water 9 µL, 1 µL forward primer, 1 µL reverse primer, 3 µL DNA template. PCR machine cycling conditions were predenaturation at 96°C for 1 minute to ensure the denaturation process. Then denaturation was carried out at 96°C for 30 seconds, annealing at 58°C for 30 seconds, and elongation at 72°C for 1.5 minutes. The last cycle at 72°C for 3 minutes, this PCR process was run for 40 cycles. Amplification products were sent to Firstbase Company in Malaysia to be purified and sequenced.

### DNA Analysis

The DNA STAR program combined forward and reverse DNA sequences (Burland 2000). Complete DNA sequences inputted to the BLAST (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/) and were compared with the other sequences. *P. t. sumatrae* and the other sequences obtained from GenBank (Table 2) were then aligned using the Clustal X version 1.8. Next, sequence divergence among samples was calculated using the MEGA (Molecular Evolutionary Genetics Analysis) program version 7 with the p-distance model (Kumar et al. 2016). Phylogenetic tree analysis using the MEGA 7

program (Molecular Evolutionary Genetics Analysis) (Kumar et al. 2016). The topology of the phylogenetic tree used four methods: 1) Neighbor-Joining (NJ) based on the Kimura 2 parameter(K2P) model; 2) Maximum Likelihood (ML) method based on K2P model with the Neighbor-Joining algorithm, and BioNJ; 3) Maximum Parsimony (MP) with the Subtree Pruning-Regrafting (SPR) algorithm, and 4) Minimum Evolution (ME) with the K2P model. We used 1000 bootstrap values to ensure the phylogenetic tree analysis.

## RESULTS AND DISCUSSION

### Result

Eighteen samples were successfully isolated, two preserved samples (PTS 2 and PTS 7) and one blood sample (PTS 13) were failed. This was because the samples obtained were too damaged and few. After amplification, the PCR product was visualized on a 0.8 % agarose gel; 17 samples were successfully amplified (Figure 1).

*Identification analysis*

All DNA sequences obtained (999 bp) were searched for similarity sequences on the NCBI by using the BLAST program of NCBI's GenBank. From 16,990 bp of mitochondrial DNA sequence, at 6280-7824 bp is CO1 sequence (Wei et al. 2011). This research analyzed 999 bp located at 6527-7487 bp in mitochondrial DNA and 247-1245 bp in the CO1 gene sequence. Based on the sequence similarities among samples in this study, all samples were shown to belong to *P. t. sumatrae*. BLAST analysis of 999 bp sequences with the *P. tigris* reference sequence (NC_010642.1) showed the similarity value was 99.60%-99.70% and 99.90%-100% with *P. t. sumatrae* (JF357969.1) (Table 3).

Sequences located at 6527-7487 bp of the *P. tigris* (NC_010642.1) complete mitochondrial DNA sequence. This data validated the *P. t. sumatrae* samples. The AT content in all sequences ranged from 56.2-58.1%, with an average of 56.4%. The GC content in all sequences ranged from 41.9 to 43.8%, with an average of 43.6%. Furthermore, out of 999 nucleotide bases, there are 819 (81.98%) conserved sites and 180 (18.02%) variables sites. Total 180 variable sites, there were 83 bases (8.31%) singleton sites and 97 bases (9.71%) parsimony sites.

*Phylogenetic analysis*

Genetic distances were analyzed that formed the basis of the reconstruction of the phylogenetic tree. Intraspecies genetic distance of *P. t. sumatrae* is 0-0.1%. The lowest genetic distance caused the short branch of the phylogenetic tree of *P. t. sumatrae* species. The interspecific genetic distance of all sequences ranged from 8.9 to 14.2% (Table 4). This genetic distance was among The Felidae family, the data were supported by Pietsch (2011) and Ashrifurrahman et al. (2019) that interspecies genetic distances in the Felidae family ranged from 8.3% to 39.9.

### Discussion

The CO1 gene successfully detected *P. t. sumatrae* species by comparing the seized sample sequences with NCBI data. The 100% similarity of the forensic samples obtained makes DNA-based identification, especially the CO1 gene, an alternative morphological identification that is limited by incomplete. In addition, identification based on the CO1 gene has also succeeded in uncovering fake tiger skin trading (Khedkar et al. 2019). The CO1 gene study has also succeeded in detecting cases of illegal trafficking in Africa (Dalton et al. 2020). Thus in this study, the genetic marker of the CO1 gene carried out can be a marker in *P. t. sumatrae* sample detection.

Phylogenetic analysis was conducted to show the relationships between species. Our topology consistently provides two main clades: clade A mentions a *P. tigris* group, clade B is the *P. pardus* group, and the *Felis catus* is an outgroup species (Figure 2). Each clade clusters into a monophyletic group, which means that each individual in these clades has a common ancestor. Monophyletic grouping based on synapomorphic characters which is a derived character contained in two or more taxa (Nei and Kumar 2000)

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

| Sequences | Authors | GenBank accession |
|---|---|---|
| *P. t. sumatrae* | Kitpipit et al. 2012 | JF357969.1 |
| *P. t. sumatrae* | Kitpipit et al. 2012 | JF357970 |
| *P. t. altaica* | Sun et al. 2015 | KF297576.1 |
| *P. t. altaica* | Zhou et al. Unpublished | MH124080.1 |
| *P. t. altaica* | Zhou et al. Unpublished | MH124110 |
| *P. t. altaica* | Shchelkanov et al. Unpublished | MN624080.1 |
| *P. t. amoyensis* | Zhang et al. 2011 | HM589215.1 |
| *P. t. corbetti* | Kitpipit et al. 2012 | JF357972.1 |
| *P. pardus* | Paijmans et al. Unpublished | MH588631 1 |
| *P. pardus* | Paijmans et al. Unpublished | MH588626 1 |
| *P. pardus* | Paijmans et al. Unpublished | MH588627 1 |
| *Felis catus* | Lopez et al. 1996 | NC_001700.1 |

**Table 3.** Sequences comparison result with the NCBI data sequences

| Sample id | Similarity% on NCBI | |
|---|---|---|
| | *Panthera tigris* (NC_010642.1) | *Panthera tigris sumatrae* (JF357969.1) |
| PTS1 | 99.70% | 100% |
| PTS3 | 99.70% | 100% |
| PTS4 | 99.70% | 100% |
| PTS5 | 99.70% | 100% |
| PTS6 | 99.70% | 100% |
| PTS8 | 99.70% | 100% |
| PTS9 | 99.70% | 100% |
| PTS10 | 99.70% | 100% |
| PTS11 | 99.70% | 100% |
| PTS12 | 99.70% | 100% |
| PTS14 | 99.60% | 99.90% |
| PTS15 | 99.60% | 99.90% |
| PTS16 | 99.60% | 99.90% |
| PTS19 | 99.70% | 100% |
| PTS20 | 99.60% | 99.90% |

**Table 4.** Genetic distances among species

| No | Code Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | *PTS1* | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **2** | *PTS3* | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **3** | *PTS4* | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | | |
| **4** | *PTS5* | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | | | | | |
| **5** | *PTS6* | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | | | | |
| **6** | *PTS8* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | | | |
| **7** | *PTS9* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | | |
| **8** | *PTS10* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | | |
| **9** | *PTS11* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | | |
| **10** | *PTS12* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | | | | | | | | | | | | | |
| **11** | *PTS14* | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | | | | | | | | | | | | | | | | |
| **12** | *PTS15* | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.000 | | | | | | | | | | | | | | | |
| **13** | *PTS16* | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.000 | 0.000 | | | | | | | | | | | | | | |
| **14** | *PTS19* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.001 | 0.001 | 0.001 | | | | | | | | | | | | | |
| **15** | *PTS20* | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.000 | 0.000 | 0.000 | 0.001 | | | | | | | | | | | | |
| **16** | *MH588627_1_P_pardus* | 0.101 | 0.101 | 0.101 | 0.101 | 0.101 | 0.101 | 0.101 | 0.101 | 0.101 | 0.101 | 0.099 | 0.099 | 0.099 | 0.101 | 0.099 | | | | | | | | | | | |
| **17** | *MH588631_1_P_pardus* | 0.094 | 0.094 | 0.094 | 0.094 | 0.094 | 0.094 | 0.094 | 0.094 | 0.094 | 0.094 | 0.093 | 0.093 | 0.093 | 0.094 | 0.093 | 0.009 | | | | | | | | | | |
| **18** | *MH588626_1_P_pardus* | 0.089 | 0.089 | 0.089 | 0.089 | 0.089 | 0.089 | 0.089 | 0.089 | 0.089 | 0.089 | 0.088 | 0.088 | 0.088 | 0.089 | 0.088 | 0.014 | 0.010 | | | | | | | | | |
| **19** | *JF357969_1_P_t_sumatrae* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.001 | 0.001 | 0.001 | 0.000 | 0.001 | 0.101 | 0.094 | 0.089 | | | | | | | | |
| **20** | *JF357970_1_P_t_sumatrae* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.001 | 0.001 | 0.001 | 0.000 | 0.001 | 0.101 | 0.094 | 0.089 | 0.000 | | | | | | | |
| **21** | *KF297576_1_P_t_altaica* | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.002 | 0.002 | 0.002 | 0.001 | 0.002 | 0.099 | 0.093 | 0.088 | 0.001 | 0.001 | | | | | | |
| **22** | *MH124110_1_P_t_altaica* | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.002 | 0.002 | 0.002 | 0.001 | 0.002 | 0.099 | 0.093 | 0.088 | 0.001 | 0.001 | 0.000 | | | | | |
| **23** | *MN624080_1_P_t_altaica* | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.002 | 0.002 | 0.002 | 0.001 | 0.002 | 0.099 | 0.093 | 0.088 | 0.001 | 0.001 | 0.000 | 0.000 | | | | |
| **24** | *JF357972_1_P_t_corbetti* | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.002 | 0.002 | 0.002 | 0.001 | 0.002 | 0.099 | 0.093 | 0.088 | 0.001 | 0.001 | 0.000 | 0.000 | 0.000 | | | |
| **25** | *MH124080_1_P_t_altaica* | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.002 | 0.002 | 0.002 | 0.001 | 0.002 | 0.099 | 0.093 | 0.088 | 0.001 | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 | | |
| **26** | *HM589215_1_P_t_amoyensis* | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.004 | 0.004 | 0.004 | 0.003 | 0.004 | 0.102 | 0.096 | 0.093 | 0.003 | 0.003 | 0.004 | 0.004 | 0.004 | 0.004 | 0.004 | |
| **27** | *NC_001700_Felis_catus* | 0.142 | 0.142 | 0.142 | 0.142 | 0.142 | 0.142 | 0.142 | 0.142 | 0.142 | 0.142 | 0.141 | 0.141 | 0.141 | 0.142 | 0.141 | 0.118 | 0.122 | 0.120 | 0.142 | 0.142 | 0.141 | 0.141 | 0.141 | 0.141 | 0.141 | 0.141 |

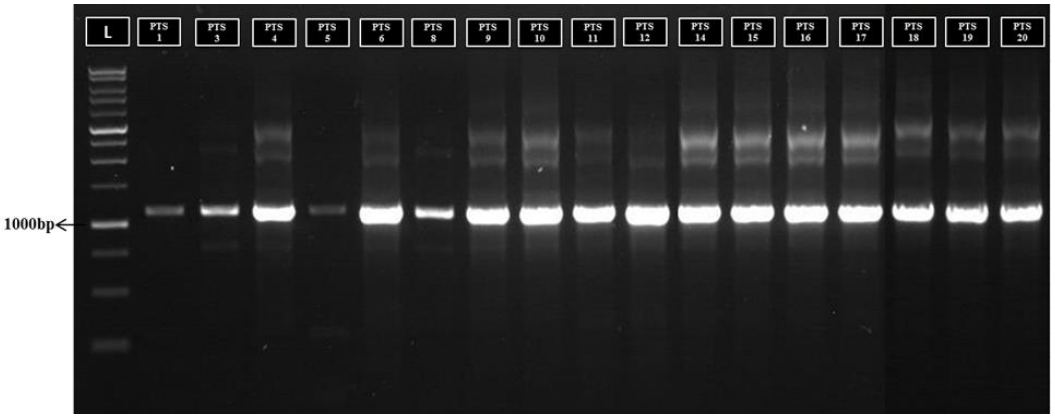


**Figure 1.** PCR result electrophoresis of the cytochrome oxidase gene subunit one mitochondrial DNA on *P. t. sumatrae* using 0.8% agarose gel. L = Ladder 1000 bp (base pair)

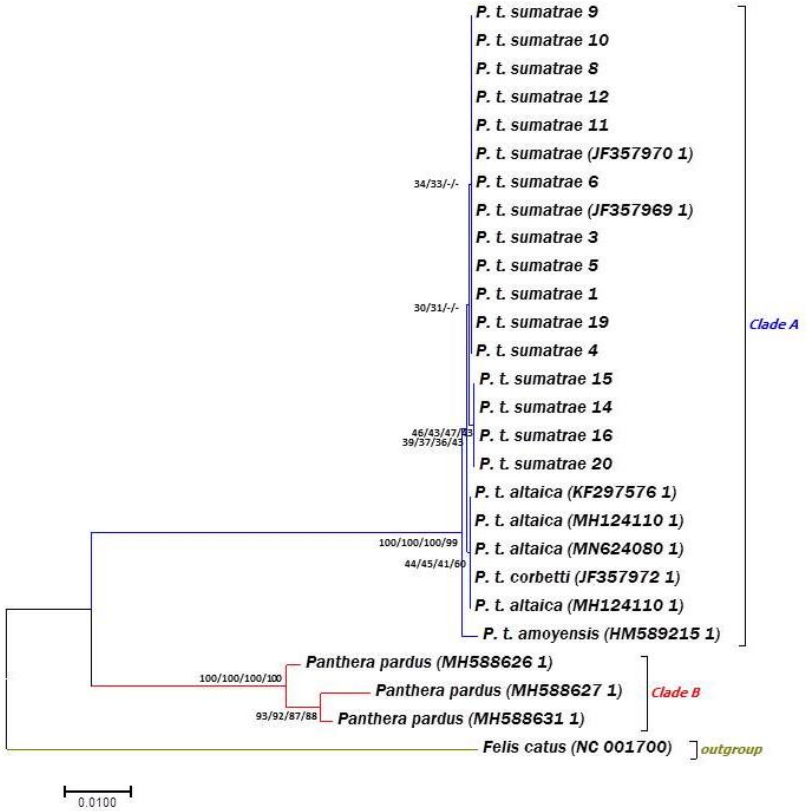


**Figure 2.** Neighbor-joining phylogenetic tree *Panthera tigris sumatrae* and comparison species based on the CO1 gene with 1000 bootstrap replicates (NJ/ME/MP/ML)

All individuals of *P. tigris* in clade A were not in a straight line, and several nodes are separating the individuals (Figure 2). The nodes divide among *P. t. amoyensis* with other subspecies (bootstrap value: 99-100%). Branching on the *P. t. amoyensis* with other subspecies shows a high genetic distance (0.3-0.4%). This subspecies is thought to have evolved earlier because exclusively found in the second branch after *P. pardus*. Wilting et al. (2015) also reported that *P. t. amoyensis* was quite far apart from other subspecies, despite being still within the same species scope in phylogenetic tree reconstruction using 3968 bp of mtDNA. *P. t. amoyensis* indicated the origin of the formation of modern tigers (Herrington 1987). The modern tiger's ancestors have been estimated to appear at 72,000-108,000 years ago (Luo et al. 2004). In comparison, the early emergence of morphology in modern tiger species was estimated to occur two million years ago in eastern China (Herrington 1987).

The distribution of tigers originated from *P. t. amoyensis* in Southern China, which assumed a separate clade that marks the beginning of the tiger's evolution (Figure 2). Current facts of tigers, *P. t. sumatrae* evolved

earlier on Sumatra and tropical Sunda Island than *P. t. altaica* in North China and *P. t. corbetti* in Indochina. This expansion and divergence process began speciation among tiger subspecies globally. The first wave of distribution of tigers originating from Indochina lasted at 110,000-70,000 years ago, in the *P. t. sumatrae* subspecies (Liu et al. 2018). This isolation was most likely related to the Toba supereruption 75,000 years ago in Sumatra (Rampino and Self 1993). The second wave distribution was estimated to have occurred 50,000-10,000 years ago in the *P. t. altaica*, *P. t. corbetti*, and *P. t. tigris* (Liu et al. 2018).

Further nodus separates *P. t. sumatrae* with *P. t. altaica* and *P. t. corbetti* with a 36-43% bootstrap value. Geographically, *P. t. sumatrae* create a group as Sunda tigers while *P. t. altaica* and *P. t. corbetti* are included in Continental tigers (Wilting et al. 2015). The branch marking that separated each subspecies was thought to have occurred in the interglacial period. The Sundanese and Chinese regions respectively began to be isolated as sea levels rose (Kitchener and Yamaguchi 2010). Branch formings on the *P. t. sumatrae* with other subspecies being a basis which reinforces that *P. t. sumatrae* and other subspecies undergo allopatric speciation currently geographically very far apart.

Lastly, the node separated the *P. t. sumatrae* with 29-31% bootstrap value (Figure 2). The branches length of each node in clade A is very short, with a bootstrap value below 50% (Figure 2). The bootstrap value is stable above 95% and be unstable below 70% (Hillis and Bull 1993). However, the existence of branching signifies differences in genetic differences between individuals. The genetic distance value of about 0-0.1% in *P. t. sumatrae* in this study indicated a gene flow process among the population. Several variables result in low or high values for genetic distance between a population or individual such as geographic isolation and reproductive isolation. Genetic distance indicates the possibility of geographical isolation affecting a population (Faizah 2008). The lower value of the genetic distance (p-distance) between a population or individual, indicates the closer the population with no barrier between them (Luo et al. 2004; Xue et al. 2015).

Using gen CO1 markers identified the illegal trade case of *P. t. sumatrae* with various sample types despite the samples found in preserved hair, nail, and bones. These results are very important because the hunting and trading of *P. t. sumatrae* parts are increasingly prevalent (Forum Harimau Kita 2019). Then, the phylogenetics of the *P. t. sumatrae* and other subspecies forms a monophyletic relationship and separating process approximately 100,000 years ago by Sumatra and mainland Asia breaking up.

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