(RESEARCH ARTICLE)

# Characteristic of CO1 gene in "Bonita", one of the female tiger *Panthera tigris sumatrae* in East Sumatra

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## Abstract

Based on the IUCN Red List, *Panthera tigris sumatrae* or Sumatran Tiger holds the status of Critical (Critically Endangered /CR) species with an estimated population of only 371 individuals in the wild. Illegal hunting and trade are one of the causes that led to population decline drastically. Conventional forensic investigation such as morphological data collection is difficult to do because the sample has been degraded or mixed with other animal body parts. DNA barcode is an actual method that utilizes DNA for species identification based on the gene markers of living things. DNA barcode usually uses a DNA nucleotide sequence of genes that is different between species and almost unchanged in that species. The gene is *cytochrome oxidase subunit 1* (CO1). Here we report the results of the characterization of the CO1 gene for DNA barcoding from one of the Sumatran tigers with an individual identity called Bonita caught in Riau province and released after being rehabilitated. This research has been able to characterize the CO1 Bonita gene along 435 nucleotides. That length of the nucleotide can be used as a Bonita barcode with the change in the eighth amino acid, Leucine to Proline with the 23rd nucleotide mutation (CTG→CCG). CO1 can distinguish individuals in intra-species, which in the eighth amino acid from other *Panthera tigris* is Leucine. CO1 gene can differentiate between species analyzed in this study with genetic distances greater than 8.3%.

**Keywords:** DNA barcoding; Felidae; NCBI; PCR

## 1. Introduction

Sumatran tiger is one of the six subspecies of *Panthera tigris* that still survive, and the only ones still exist in Indonesia. Sumatran tigers are a natural predator, but their population has declined [1]. In 1978 the population estimated were about 1,000 individuals [2]. In 1990 it was estimated that 400-500 individuals left in the wild [3]. The declining of the Sumatran tiger populations caused by several factors, including the increase of illegal trade and human conflict [4]. Based on data from South Korean Customs, TRAFFIC Species in Danger Report between 1970-1993, 8.981 kg tiger bones found smuggled into South Korea, and more than (> 44% (3.994 kg) came from Indonesia [5].

The illegal trade of the body parts of Sumatran tiger continues, evidenced by revealed Sumatran tiger trade activities in Riau and Medan Province in 2016 [6]. It is estimated that there are still many cases of persecution and illegal trade that cannot be disclosed. Samples of body parts of the Sumatran Tiger traded on the black market are sometimes challenging to identify. Some constraints are the conventional methods of identification and forensics. Standard methods based on morphology and immunology have limitations in the identification of samples that have been old and degraded [7]. For this reason, it is necessary to use the other identification method.

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Characterization is an activity to identify the essential characteristics of the particular species. One of the characters observed can be in the form of molecular aspects. The latest DNA-based identification method is developed for animal forensic investigation [8, 9] and known as DNA barcoding technology, which was established in 2003 [10, 11]. Currently, DNA barcoding has been used as a standard protocol for the rapid identification of animals and plants at the species level. DNA barcode consists of short pieces of DNA from standard genome regions. The cytochrome oxidase subunit 1 (CO1) gene is the gene used as the standard barcode for almost all groups of animals. The standard region of this gene in the mitochondrial is around 650 base pairs. CO1 has proven to be very useful in identifying birds, butterflies, fish, flies, and many other animal groups. The advantage of using a CO1 is that parts of the gene are short enough. The gene sorted quickly and cheaply to identify variations between species, very few deletions and insertions in the sequence, and mostly are conserved [10]. The DNA barcode is the sequence that can be used to identify different species. The barcode is the same way as a supermarket's scanner with the typical black lines of the Universal Product Code (UPC) that uses to identify our purchases.

One of the individuals at Sumatran Tiger Rehabilitation Center (PR-HSD) is a female with an individual identity called Bonita. Bonita was rehabilitated because of a conflict with humans in Riau, East Sumatra. After being rehabilitated, Bonita has been released to the jungle. At the same time, while Bonita is prepared to be released, we do the study to characterize DNA from Bonita's blood sample. The results of this study can be used to DNA barcode reference of other Sumatran Tigers especial for the forensic database to reveal the illegal trade.

## 2. Material and methods

### 2.1. Sample sources and DNA extraction

A blood sample of Bonita was collected at Sumatran Tiger Rehabilitation Center in Dharmasraya (PR-HSD). Bonita's blood put into a 1.5 ml voculab tube. The samples were stored in -20oC. DNA extraction from blood sample following DNA Invitrogen PureLink ™ Genomic DNA Kit protocol. The DNA extract was stored at -20oC for further analysis.

### 2.2. Polymerase chain reaction (PCR) and DNA sequencing

Mitochondrial DNA CO1 segment was amplified with PCR using primers: forward C11020F (5' CCAGAAGTCTATATCTTAATCCCG 3') and reverse T11428R (5' GCTCCTATTGACAAG ACGTAG 3') [12]. DNA amplification was performed in 25 µl reaction mixture containing: 11µl Bioline master mix, 9µl nuclease free water, 1µl forward primer, 1µl reverse primer, and 3µl DNA template. The PCR process was set as follow:  an initial denaturation at 96oC for 1.0 min, 35 cycles of denaturation at 96oC for 0.5 min; annealing at 50oC for 0.5 min; extension at 72oC for 1.5 min; and final step at 72oC for 3.0 min. PCR product was visualized on 1.2% agarose gels with ethidium bromide staining. The most powerful PCR product was sent to Firstbase Company Malaysia for nucleotide sequencing reactions.

### 2.3. DNA sequence analysis

The nucleotide sequence of the CO1 gene of Bonita was assembled and edited by visual examination with the DNA STAR program [13].

**Table 1** List of comparative species

| No | Species | Authors | GenBank Accession |
|----|---------|---------|-------------------|
| 1 | *Panthera tigris sumatrae* 1 | [17] | JF357969.1 |
| 2 | *Panthera tigris sumatrae* 2 | [17] | JF357970 |
| 3 | *Panthera tigris altaica* | [18 | MH124112.1 |
| 4 | *Panthera tigris amoyensis* 1 | [19] | HM589214 |
| 5 | *Panthera tigris tigris* | [20] | JF357967 |
| 6 | *Panthera tigris amoyensis* 2 | [19] | NC_014770 |
| 7 | *Panthera tigris corbetti* | [20] | JF357971 |
| 8 | *Panthera leo* | [21] | KF907306.1 |
| 9 | *Panthera onco* | [22] | KF483864.1 |
| 10 | *Prionailurus bengalensis* | [23] | KX857771.1 |
| 11 | *Prionailurus viverrinus* | [24] | KR135742.1 |

The DNA sequence was compared with the 11 sequences sampled (Table 1) from GenBank NCBI https://blast.ncbi.nlm.nih.gov/Blast.cgi using the Basic Local Alignment Search Tool (BLAST) program to measure the degree of data similarity. All of the sequences were edited using BioEdit program [14] and aligned using the Clustal X [15]. Data analysis using MEGA program version 7, and the genetic distances computed by applying the Kimura 2-parameter (K2P) model of sequence evolution [16]. DNA barcode obtained by entering the CO1 sequence into the Bio-Rad DNA Barcode Generator site (biorad-ads.com/DNABarcodeWeb/).

## 3. Results and discussion

### 3.1. Amplification fragment gene cytochrome oxidase subunit 1 (CO1)

The results of the total DNA extraction were migrated to 1.2% agarose gel and viewed with UV trans-illuminator rays. The total DNA was used as a DNA template to amplification the CO1 gene in the PCR process. Amplification of the mtDNA CO1 region in Bonita's blood sample resulted in a single band measuring around 500 bp. Based on [17] the size of DNA fragments *Panthera tigris sumatrae* CO1 gene is 957 bp, 657 bp in *Panthera tigris* [12], and 648 bp in *Panthera tigris amoyensis* [19].

### 3.2. BLAST analysis and alignment

The similarity of Bonita's CO1 gene DNA sequence with GenBank data was analyzed with BLAST software. The results of the databases were used to assess the difference in nucleotide divergence within and between closely related species. The result showed that Bonita's sequence has a 99% similarity with other *Panthera tigris sumatrae.* The DNA sequence of CO1 genes Bonita was 435 bp, occupies the complete mitochondrial genome of *Panthera tigris sumatrae* at position 6071-6505 bp. In line with the study of [20] who reported that the CO1 gene of *Panthera tigris sumatrae* in mitochondrial DNA complete genome was at position 5351-6895 bp. The position of Bonita's sequence was in the 720 bp to 1155 bp of the CO1 gene of *Panthera tigris sumatrae* species.

The total of 12 total sequence analyzed showed that there were 371 bp (85.29%) conserved site and 64 bp (14.71%) site variable that consists of 53 bp (12.18%) parsimony site and 11 bp (2.53%) singleton site. The composition of Bonita nucleotide bases were A (Adenine) 26%, T (Thymine) 31.3%, G (Guanine) 20.7% and C (Cytosine) 22.1%. Thymine is a dominant base of the CO1 gene of Bonita. The nucleotide base of Adenine + Thymine (A + T) was 57.9% while the nucleotide base of Guanin + Cytosine (G + C) was 42.1%. According to [25], the base of G + C in the vertebrate group was lower than the A + T base.

### 3.3. Sequence and amino acid variation

Molecular evolutionary genetics analysis (MEGA) version 7.0 software was used to translate all nucleotide bases from the sequenced of the CO1 gene into amino acids. Based on the results of amino acid translations, it is known that all codons translated to 145 amino acids with changes in four amino acids at the 8th, 16th, 46th, and 131st (Table 2). One of them, the 8th amino acid in Bonita individuals which Leucine (CTG) changes to Proline (CCG). The mutation CO1 sequence that made Leucine becomes Proline also was reported by [26] in *Nycticebus* species. The change occurred because of a missense mutation. According to [27], missense mutation occurs if the substitution of nucleotide bases obtained a new amino acid translation.

Table 2 also showed that the amino acid of *Panthera onco* was changed where Histidine (CAT) become Arginine (CGT). Kind of that mutation is a transversion where the purine nucleotide base changes to purine. *Panthera leo* had two changes in amino acids, first was transversion mutation that causes the conversion of the amino acid Isoleucine (ATC) to Serine (AGC) and second was transition mutations with the change of the amino acid Tyrosine (TAC) to Cysteine (TGC). The difference in the eighth amino acids in Bonita can make the characteristics of Bonita compared to other Panthera tigris. In overall, the composition of amino acids of the Bonita CO1 gene and the related species change rarely.

**Table 2** Variation of Bonita CO I amino acids and 11 comparative species

| No | Spesies | L 8 | H 16 | I 46 | Y 131 |
|----|---------|-----|------|------|-------|
| 1 | *Panthera  tigris altaica* | . | . | . | . |
| 2 | *Panthera  tigris tigris* | . | . | . | . |
| 3 | *Panthera  tigris sumatrae 1* | . | . | . | . |
| 4 | *Panthera  tigris sumatrae 2* | . | . | . | . |
| 5 | *Panthera  tigris amoyensis* | . | . | . | . |
| 6 | *Panthera  tigris corbetti* | . | . | . | . |
| 7 | *Panthera  tigris amoyensis* | . | . | . | . |
| 8 | *Prionailurus bengalensis* | . | . | . | . |
| 9 | *Prionailurus viverrinus* | . | . | . | . |
| 10 | *Panthera  leo* | . | . | S | C |
| 11 | *Panthera  onco* | . | R | . | . |
| 12 | Bonita B1 | P | . | . | . |

## 3.4. Phylogenetic analysis

Phylogenetic analysis with distance genetic inter sequences can be used to show the relationship between analyzed species.  The result of the phylogenetic tree for all sequence that made by using MEGA 7.0 program [16]. The tree was constructed by combining Maximum Likelihood (ML), Neighbour-joining (NJ), Minimum Evolution (ME) and Maximum Parsimony (MP) methods with 1000 replicate bootstraping (Figure 2).
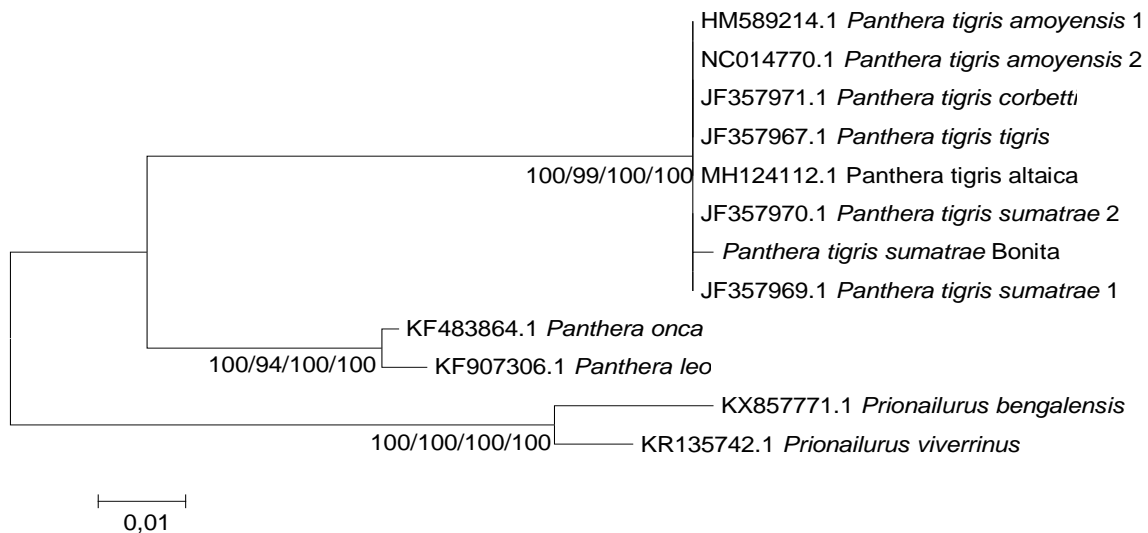


**Figure 1** The ML phylogenetic tree of the CO1 gene with bootstrapping of 1000 replicates (ME/ML/NJ/MP)

The phylogenetic tree (Figure 1.) showed that there were two main clusters obtained, *P. tigris* group and other *P. tigris* group (*P. onca* and *P. leo*) supported by highly bootstrap value 100/99/100/100 for ME/ML/MP, respectively. The First group consists of Bonita and the other *P. tigris* with 0.0-0.2% sequence divergence. Referring to the research of [28], which states that individuals in the Felidae family were at the same species-level if they had a genetic distance was about 0%-2.32%. Therefore Bonita and member of *P. tigris* is differing in individual genetic variation level.

The sequence divergence between *P. tigris* and another group (*P. onca* and *P. leo*) were 8.3-8.8%. According to previous research [28],  those value indicated that they are in different species in the same genus. The highest sequence divergence was between Bonita with *Prionailurus bengalensis* and *Prionailurus viverrinus,* which had 12.2 % and 12.5%

sequence divergences. Referring to [28], different species in the same Family in Felidae have a genetic distance of 10.3%-39.9%. From the present study, we obtained the DNA Barcode of Bonita (*P. tigris sumatrae*). The sequence consists of 435 base pair of cytochrome oxidase subunit 1 (CO1) DNA mitochondrial gene. It has a specific site at 23rd nucleotide position with changed of thymine to cytosine as well as Leucine became Proline at the eighth sequence of its amino acid.

## 4. Conclusion

From a total of 435 bp in the CO1 gene, we obtained Bonita DNA Barcode and the specific site in the 23rd sequence nucleotide base with the change of thymine to cytosine.

## Compliance with ethical standards

### Disclosure of conflict of interest

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

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