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The Vulnerable Fishing Cat *Prionailurus viverrinus* from Wonorejo Mangroves, Surabaya, Indonesia Based on Morphology and Molecular Data

Sunu Kuntjoro*[1], Dwi Anggorowati Rahayu[2], Widowati Budijastuti[3], Winarsih[4]

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[1,2,3,4]Biology Study Program, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya, Jl. Ketintang, Surabaya City, East Java, Indonesia, 60231

e-mail:
[1*]*sunukuntjoro@unesa.ac.id*
[2]*dwirahayu@unesa.ac.id*
[3]*widowatibudijastuti@unesa.ac.id*
[4] *winarsih@unesa.ac.id*

**Corresponding author*

Abstract. *The fishing cat (Prionailurus viverrinus) is a protected animal. Currently, the population in nature is declining due to over-fishing and changes in environmental quality. The existence of this fishing cat is an interesting finding that must be immediately studied at the morphological and genetic levels for certainty of its species. This study aimed to identify fishing cat from Wonorejo Mangroves, based on morphology and Cyt b genes analysis. The results of Cyt b identification obtained a DNA sequence length of 398 bp with a similarity value of the five Prionailurus sp. samples between 96.75 to 98.97%. The identification using molecular data consisted of five variations of nucleotide bases, and the average value of the genetic distance with the ingroup was 1.0%., The Cyt b DNA markers analysis succesfully identified fishing cat from Wonorejo Mangroves as Prionailurus viverrinus.*

Keywords: *cyt b, genetic distance, molecular data, morphology, phylogenetic*

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INTRODUCTION

One of Indonesia's biodiversity which has attractiveness and potential in various ecosystems, is rare, and valuable in conservation, and its constituent is Mangrove. Mangroves are ecosystems that have intertidal regions, where there is a unique interaction between marine, brackish, river, and terrestrial waters. This interaction makes the mangrove ecosystem have a high diversity of both flora and fauna (Kaliu et al., 2013; Martuti, 2013; Zakaria et al., 2015; Rout et al., 2017; Sari et al., 2019). One of the unique vertebrates that is interesting to study in more detail is the fishing cat (*Prionailurus* sp.) whose existence is suspected through the results of camera traps in the Wonorejo Mangroves, Surabaya.

The fishing cat (*Prionailurus* sp.) is the largest cat of the other Prionailurus cats. The fishing cat is twice the size of the domestic cat. An elongated face with a characteristic flat nose. Underparts are white, and behind ears are black with central white spots (Ko et al., 2022; Patel et al., 2017; Teng et al., 2022). The existence of this fishing cat is an interesting finding that must be immediately studied at the morphological and genetic levels for the certainty of its type. Based on Government Regulation No. 7 of 1999, there are six species of cats that are protected, including the mangrove cat (*Prionailurus viverrinus*). The certainty of the type of a species can be detected based on morphology (Haryono et al., 2006; Page et al., 2020; Basith et al., 2021) and strengthened by molecular markers (Sem-

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biring et al., 2015; Mikkelsen et al., 2017; Anzani et al., 2019; Letchuman, 2018; Puillandre et al., 2021).

The Cytochrome b (Cyt b) gene has characteristics that can be used in determining species identity in almost all higher fauna. The Cyt b gene undergoes very few deletions and insertions in its sequence, it has many conserved genes, making it suitable for conducting DNA barcoding in most species.. Moreover, the Cyt b gene is often used to compare multiple phylogenetic species in the same genus or family. The diversity of the Cyt b gene has been used to detect the source of milk derived from cattle (Bos), sheep (Ovis), goats (Capra), and buffalo (Bubalus) (Lanzilao et al., 2005)

Based on the previous, scientific information regarding the identification of *Prionailurus* sp. found at Wonorejo Mangroves is necessary. The identification used is molecular markers, especially the Cyt b gene, to reinforce the morphological data. In addition, analyzing phylogenetics to determine the relationships between *Prionailurus* sp. with relatives from Genbank NCBI (*National Center for Biotechnology Information*) is also very important. Therefore, the study aimed to identify *Prionailurus* sp. from Wonorejo Mangroves, using a molecular marker, namely the Cyt b gene to characterize the biodiversity and conservation of fishing cat in East Java, Indonesia.

MATERIALS AND METHODS

This research was conducted from March to June 2023. This research was carried out in the Wonorejo mangrove forest.

Study Area

The sampling location was chosen based on an initial survey that had been carried out through the alleged camera trap results. The Wonorejo mangrove map based on the geographic information system (GIS) is presented in Figure 1.

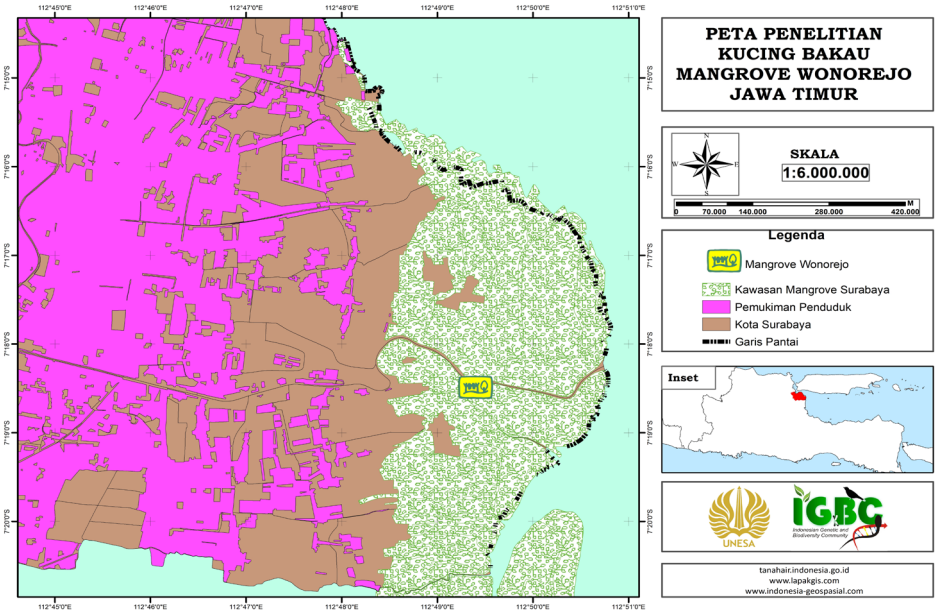

Figure 1. Sampling locations on Wonorejo Mangrove Forest

Sampling Technique

This research was carried out with the assistance of the Surabaya City Agriculture Service and blood samples were taken together with a veterinarian from this agency. Cats caught were immediately anesthetized to have their blood drawn and released back into the wild.

DNA Isolation

DNA isolation was usedNEXprep Cell/ Tissue DNA Mini Kit. A 200 µl blood sample collection tube was added to 200 µl Buffer GT1 and homogenized using a vortex. First, the lysis stage was carried out by adding 200 µl Buffer GT2 and 20 µl Proteinase K into the sample and mixing it using a vortex. Next, the binding stage was conducted by adding 200 µl of absolute ethanol to the sample and mixing it with a vortex. Then, the sample was put into the spin column and was centrifuged at 13,000 rpm for 1 minute. Purification was conducted by adding500 µl of Buffer W1 to the spin column and centrifuging at 13,000 rpm for 1 minute. Then, the flow through is removed and the high filter tube is reattached to the collection tube. Next, 700 µl of Buffer W2 was added to the spin column and centrifuge at 13,000 rpm for 1 minute. Then the flow through was discarded and the high filter tube was attached back to the collection tube and centrifuge at 13,000 rpm for 2 minutes. Finally, the elution stage was carried out by adding 100 µl of Elution Buffer and the DNA sample was incubated simultaneously at 70°C for 1 minute and then centrifuged at 13,000 rpm for 1 minute. The DNA obtained was then stored at -20ºC.

Amplification Cytb Gene

DNA amplification was conducted using PCR by utilizing a pair of primers with the Cyt b gene target L14841 5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3' and H15149 (5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3') (Kocher et al., 1989). The PCR method used was the hotstart method using PCR master mix (2x Mytaq HS Red Mix). A repeating principle starts with denaturation, annealing, and extension which was carried out with an amplification cycle of 40 cycles to get a good PCR quality without a smear. Each cycle consisted of a double thread attachment process (pre-denaturation) at 94°C for 1 minute, a denaturation at 94°C for 45 seconds, annealing at 50°C for 1 minute, and an extension at 72°C for 2 minutes. Then it proceeded further, with a final extension at 72°C for 10 minutes.

Electrophoresis

Electrophoresis medium for the results of DNA isolation was prepared with a 1% agarose gel composition (0.2 g agarose and 20 mL 0.5x TBE) mixed using a magnetic stirrer for 5 minutes. The mixture was then put into the gel slab and waited for 20 minutes. The result of the gel slab was put into the electrophoresis chamber and 10x TBE was poured into the electrophoresis chamber until it reached a height of 1 mm above the gel. A 3 µL PCR product was mixed with 2 µL loading dye NEXview Nucleic Acid Stain and 1 µl Solution Distilled Water (SDW) on parafilm paper, and was then put into the agarose well. The length of the DNA base strands was compared with a 4µL bp Lowmass ladder of 100 bp that was inserted into the agarose. Next, the machine was performed the electrophoresis with a voltage of 48 V and a current of 0.5 A for 20 minutes.

The electrophoretic medium for the PCR results was prepared with a 1.5% agarose gel composition (0.3 g agarose and 30 mL 0.5x TBE) mixed using a magnetic stirrer for 5 minutes. The mixture was put into the

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gel mold and wait for 20 minutes. The result of the gel mold was put into the electrophoresis chamber and 10x TBE was poured into the electrophoresis chamber until it reached a height of 1 mm above the gel. The next step was to mix 3 µL of the amplified DNA with 2 µL of loading dye NEXview Nucleic Acid Stain and 1 µl of Solution Distilled Water (SDW) on parafilm paper to balance the reaction with a total of 6 µl. This mixture was then put into the agarose wells. The length of the DNA base strands was compared with a 4µL bp Lowmass ladder of 100bp.The sample band was produced by having the characteristics of a single and thick band.

DNA Sequencing

DNA sequencing in the target area of the COI gene was carried out by First Base, Malaysia using the Sanger method (1977). This DNA sequenced was a pair of primers L14841 5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3' and H15149 (5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'). The results of sequencing data were base sequences in ABI chromatogram format.

Bioinformatic Analysis

The DNA sequencing results were analyze by chromatogram, using Finch TV, and translated proteins online via the Expasy web (https://web.expasy.org/translate). Sequence alignment was conducted between the study sample and the reference sequence from Genbank NCBI using Clustal X version 2.1. The final alignment was determined using Bioedit version 7.0.5.3 to analyze nucleotide base composition on research samples and variations of nucleotide bases between the samples with data from Genbank NCBI. Compilation of phylogenetic tree reconstruction used MEGA 6.0 software to obtain genetic distances and phylogenetic tree. The genetic distance was calculated using the Kimura-2 parameter (K2P) model to obtain the matrix calculation. Phylogenetic tree reconstruction was analyzed using the Neighbor-Joining Tree (NJ) and Maximum Likelihood (ML) methods with a bootstrap value of 1000 replications. Finally, the similarity values were calculated: similarity percentage = (1-Genetic distance) x 100%.

RESULTS AND DISCUSSION

Systematics and Description

The fur of the fishing cat is a rich yellowish-grey with black lines and patches. There are two stripes on the cheekbones and two above the eyes that go to the neck, with broken lines on the forehead. Around the throat, there are two rows of dots. The shoulder spots are longitudinal, whereas the dots on the sides, limbs, and tail are roundish. The belly fur is lighter than the fur on the back and sides. The short, rounded ears are situated low on the head, with a white spot on the rear. The tail is short, measuring less than half the length of the head and body and ending with a few black rings. Its paws are less totally webbed than those of other species (Figure 2).

Collection number : 1, 2, 3, 4, 5
Habitat : Wonorejo Mangrove

Figure 2. Fishing cat from Wonorejo Mangrove

Visualization of DNA

Cyt b gene target DNA amplification was performed using universal primers, namely L14841 5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3' and H15149 (5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'). The Cyt b gene targets DNA amplification results were then electrophoresed using 1.5% agarose gel and visualized with a UV-transilluminator. A well-amplified COI gene target was indicated by the presence of thick DNA bands and no smears, with DNA visualization results obtained of ±389 bp (Figure 3).

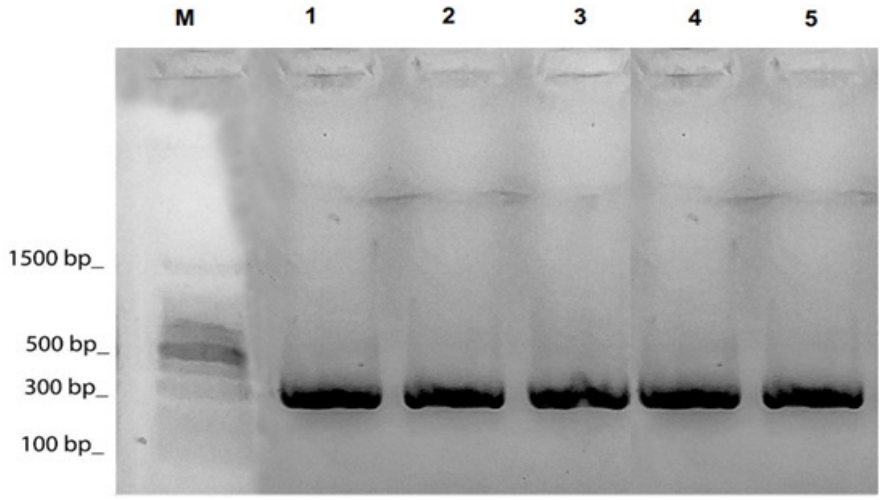

Figure 3. Visualization of DNA specimens of Prionailurus sp. from Wonorejo Mangroves, in a 1.5% agarose gel with a 100bp DNA ladder. (Image captions: M: Marker, 1-5: Prionailurus sp. 1-5).

Identification Similarity Using The BLAST

In the nucleotide base of *Prionailurus* sp. samples that have been identified in Wonorejo Mangroveswas a 398 bp sequencing length. These sequencing results were processed using Expasy through translation into protein until no stop codon was found in the middle of the nucleotide base. The results of Expasy were then analyzed using the BOLD System (Table 1) to determine the highest degree of relationship of the research samples. The analysis showed similarities between the three *Prionailurus* sp. samples with *Prionailurus viverrinus* with 99.0-99.2%. This similarity value indicates that these three samples have been identified at the species level. Therefore, it concluded that species similarities exist between the five Prionailurus sp. samples with Prionailurus viverrinus.

Composition of Nucleotide Bases

Based on the alignment phases of all samples (research samples with Genbank data), a sequencing length of 398 bp was obtained. Cyt b *Prionailurus* sp. gene barcode sequence data. The average G+C nucleotide base composition was 38.8% among the five samples, while the average A+T base composition was 61.2% (Table 1). According to these average results, the composition of the nucleotide bases of G+C was lower than the composition of the nucleotide bases of A+T (23).

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Table 1. The nucleotide base composition of Prionailurus sp. from Wonorejo Mangrove

Name Species	A (%)	C (%)	G (%)	T (%)	A+T (%)	G+C (%)
Prionailurus viverrinus 1	20.9	19.9	21.6	37.7	58.6	41.4
Prionailurus viverrinus 2	21.2	19.9	21.9	37.0	58.2	41.8
Prionailurus viverrinus 3	21.2	19.9	21.9	37.0	58.2	41.7
Prionailurus viverrinus 4	21.1	19.9	21.8	37.2	58.3	41.7
Prionailurus viverrinus 5	21.1	19.9	21.8	37.2	58.3	41.7

Variation of Nucleotide Bases

Based on nucleotide base variation, there are five patterns: transversion and transition. The changes in nucleotide bases revealed that four automorphic nucleotide base patterns were only seen in *Prionailurus* sp. samples. Variations in nucleotide base numbers 22 (thymine), 50 (thymine), and 284 (cytosine) were discovered. *Prionailurus* sp. is the sole species with automorphic nucleotide bases (Table 2). According to Rahayu & Jannah (2019) certain species only have automorphic nucleotide bases as a marker or distinguishing feature between the species and the species being compared.

Table 2. Prionailurus sp. nucleotide base variations, based on the Cyt b gene.

Species	22	42	46	50	83	284
MN370577.1 *Prionailurus viverrinus*	A	G	T	A	G	A
Prionailurus viverrinus 4 MW Surabaya	T	•	•	•	•	•
Prionailurus viverrinus 5 MW Surabaya	T	•	•	•	•	•
Prionailurus viverrinus 3 MW Surabaya	T	•	•	•	•	C
Prionailurus viverrinus 2 MW Surabaya	T	•	•	T	•	C
Prionailurus viverrinus 1 MW Surabaya	T	•	•	T	•	•
MN370578.1 *Prionailurus viverrinus*	•	•	•	•	•	•
MN370576.1 *Prionailurus viverrinus*	•	•	•	•	•	•
MN370566.1 *Prionailurus bengalensis*	T	A	C	C	C	•
MN370567.1 *Prionailurus bengalensis*	T	A	C	C	C	•

Genetic Distance

The final alignment results were processed using MEGA6 to determine the genetic distance of *Prionailurus* sp. with their relatives using the Kimura-2 parameter model calculation. The genetic distance matrix results were obtained and shown in Table 4. The average genetic distance of the *Prionailurus* sp. from Wonorejo Mangroves was 1.00%, while the average genetic distance between the study sample and the ingroup was 1.00%. In addition, the average genetic distance between the study sample and the outgroup was 6.00%. To establish the close genetic between individuals, genetic distance values were obtained using the Kimura-2 parameter model computation. The Kimura-2 calculations are used in the parameter model analysis to as-

sess the mutation's transition and transversion substitution point (Hajibabei et al., 2007). The number of nucleotide base changes influences the genetic distance (Kocher et al., 1989; Ketchum, 2016). According to Hebert et al. (2005), the distance is defined by the number of bases that change. The more variations, the more frequently the mutation process happens, indicating the further distance.

Reconstruction of Phylogenetic Trees

Phylogenetic tree reconstruction was performed using the Neighbor-Joining Tree (NJT) and Maximum Likelihood (ML) methods to obtain a phylogenetic tree for the sample *Prionailurus* sp. from Wonorejo Mangrove with data from Genbank NCBI (Figures 4 and 5). The Neighbor-Joining Tree (NJT) method results show four clusters (Figure 4). The results are as follows: Cluster ingroup I consisted of Prionailurus sp. from Wonorejo Mangrove and ingroup species. Cluster 2 in other species, *Prionailurus bengalensis*. Furthermore, based on (Figure 4) shows that the Prionailurus sp. from Wonorejo Mangrove forms the same clade as Prionailurus viverrinus and forms a bootstrap value of 71-100. Phylogenetics is a taxonomic classification method that determines diversity by reconstructing genetic distance (Popa et al., 2007). MEGA6 was used to examine the research sample's relationship with Genbank NCBI data using the final alignment data. The bootstrap values can show the stability of the derived phylogenetic tree branches (Popa et al.2007). Furthermore, bootstrap methodology is used to verify the validity of sequence data for branch placement in a phylogenetic tree (Hajibabei et al., 2007).

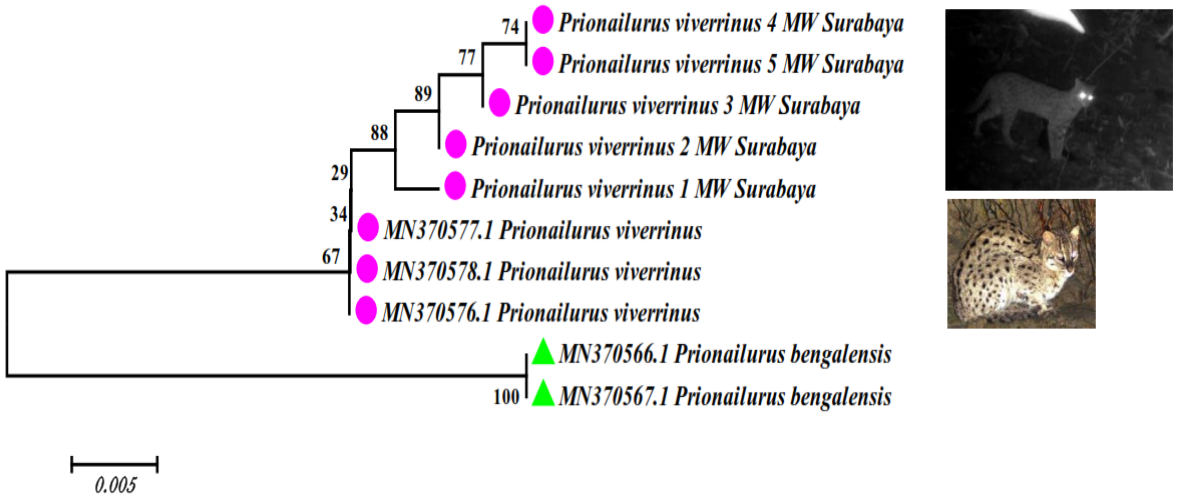

Figure 4. The phylogenetic topology Prionailurus sp. from Wonorejo Mangrove, concerning the Cyt b gene from Genbank NCBI using the Neighbor-Joining Tree (NJT) method with a bootstrap of 1000 replications.

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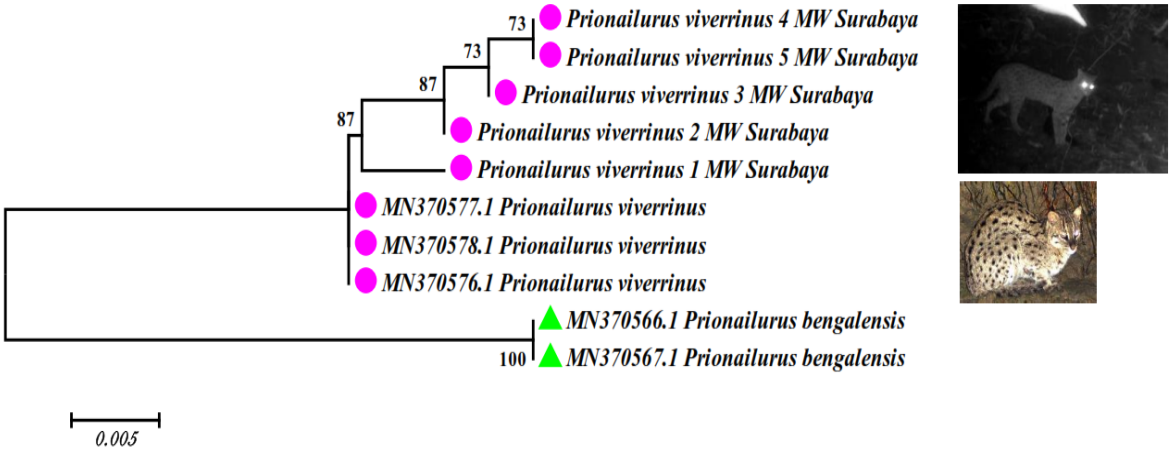

Figure 5. The phylogenetic topology Prionailurus sp. from Wonorejo Mangrove, concerning the Cyt b gene from Genbank NCBI using the Maximum Likelihood Tree (ML) method with a bootstrap of 1000 replications.

CONCLUSION

The results of Cyt b identification obtained a DNA sequence length of 398 bp with a similarity value of the five *Prionailurus* sp. samples between 96.75 to 98.97%. The identification using molecular data, consisted of five variations of nucleotide bases, and the average value of the genetic distance with the ingroup was 1.0%. *Prionailurus* sp. phylogenetic tree from Wonorejo Mangrove was in the same clade as *Prionailurus viverrinus* with the Neighbor-Joining Tree and Maximum Likelihood methods with bootstrap values between 87-100.

AUTHOR CONTRIBUTION

M.I. designed the research and supervised all the process, L.A. collected and analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

No conflict of interest regarding the research or the research funding.

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