

PHYLOGENETIC POSITION OF LONG-TAILED MACAQUE (*Macaca fascicularis*) IN PULAU TINGGI, JOHOR, MALAYSIA

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Abstract: *Macaca fascicularis* is one of the most common primates found in Malaysia; however, they are under-appreciated and under-studied, especially in the taxonomic field. The subspecies *M. fascicularis laeta* was previously hypothesized to be distributed in Pulau Tioman and Pulau Tinggi. Thus, in this study, we aim to verify the phylogenetic position of long-tailed macaque in Pulau Tinggi through a molecular approach based on the D-loop region of mitochondrial DNA using Neighbour-Joining (NJ), Maximum Parsimony (MP) and Bayesian Inference. Three samples were successfully analyzed with a 99% bootstrap value in NJ, 99% bootstrap value in MP and 1.00 posterior probability in Bayesian Inference for Pulau Tinggi's clade. The phylogenetic analysis concluded that Pulau Tinggi's population is similar to the mainland population, which is the subspecies *M. f. fascicularis*, with an estimated time of divergence at ~0.299 million years ago. Overall, this study generally provides baseline data to aid in the future translocation process and conservation management of *M. fascicularis* in Pulau Tinggi and Malaysia.

Keywords: Cercopithecidae, cercopithecinae, primate, macaque, pest, Seribuat Archipelago, South China Sea.

Introduction

Long-tailed macaque (*Macaca fascicularis*), known as 'kera' in Malaysia is one of the most successful and widely distributed non-human primates ranging from three to five million individuals in Asia alone (Gumert, 2011). This species is also known by several other names such as crab-eating macaque (Fooden, 1995). They are well-adapted and can be easily found across Southeast Asia in Malaysia, Brunei, Cambodia, Nicobar Islands, Indonesia, Laos, Myanmar, Philippines, Singapore, Thailand, Timor-Leste and Vietnam (Gumert, 2011). Two other species from the genus *Macaca* can be found in Malaysia: *Macaca nemestrina* and *Macaca arctoides* (Groves, 2001; Roos *et al.*, 2014). The morphological characteristics of *M. fascicularis* that differentiate this species from

the other two species are their significantly longer tail that usually exceeds their length from head to rump (Groves, 2001). This social animal is primarily arboreal and will use its tails for balancing to leap between the trees in the forest, even up to five meters away (Rowe, 1996).

The fact that this primate is an opportunistic species; they can be found naturally dominating various kinds of habitats such as primary and secondary forests as well as riverine, swamps, mangroves, coastal areas as well as human settlements (Fooden, 1995; Rowe, 1996; Fooden, 2006). Their current population status in Malaysia is still unidentified as studies and surveys from their widely dispersed populations make it hard to pinpoint the exact and latest figures (Abdul-Latiff *et al.*, 2017a). A study by Karuppannan *et al.* (2014) shows that there

are around 133,403 individuals distributed throughout Peninsular Malaysia. Recent IUCN Red List Status (2022) has listed this species as endangered (EN) due to significant population declines. In Malaysia, *M. fascicularis* recorded the highest human-wildlife conflict beginning since the early 1970s. The conventional practices of translocation by the authorities to resolve human-wildlife conflicts for this species remain the main challenge to conserving their genetic pool (Eudey, 2008; Abdul-Latiff et al., 2017a).

Despite that, some of the proposed subspecies of *M. fascicularis* are still understudied such as *M. f. laeta* (Elliot, 1909). Based on Medway (1969), *M. f. laeta* can be found in Pulau Tioman, Pahang and Pulau Tinggi, Johor. Abdul-Latiff et al. (2014b) has shown that the classification of *M. f. laeta* in Pulau Tioman cannot be supported based on phylogenetic analysis as it is similar to *M. f. fascicularis* in mainland Peninsular Malaysia. However, the study by Abdul-Latiff et al. (2014b) did not include Pulau Tinggi, Johor representatives. Thus, it is crucial to assess the taxonomic position of the population in Pulau Tinggi as it plays an important role in managing long-tailed macaque, ensuring a healthy population and preserving the uniqueness of their gene pool. Hence, this study aims to confirm the phylogenetic position of *M. f. laeta* in Pulau Tinggi, Johor.

Materials and Methods

The sampling was conducted in Pulau Tinggi, covering three areas: The village, the primary and secondary forest, and the rocky shore. Faecal samples were collected by following the long-tailed macaque on the island. The DNA samples were stored in 95% ethanol to preserve the DNA and subsequently stored at -20°C (Table 1).

Genomic DNA was extracted using QIAamp DNA Stool Mini Kit for faecal samples. The D-loop region of the mitochondrial DNA (mtDNA) sequence was chosen since it is a hypervariable region that is proven effective in studying the phylogenetic relationship at the population level (Abdul-Latiff et al., 2014b; 2017b). A 1,100 bp fragment of mtDNA D-loop was amplified through a polymerase chain reaction (PCR) using primers as shown in Table 2. PCR Phusion kit (New England Bio Labs) was used to amplify DNA. PCR was conducted with the following parameters: Initial denaturation for 3 minutes at 95°C, followed by 30 cycles of denaturation for 15 seconds at 95°C, annealing for 30 seconds at 52°C, elongation for 10 seconds at 72°C and a final extension stage for 10 minutes at 72°C. PCR product was subsequently sent to Apical Scientific Sdn. Bhd. in Shah Alam, Selangor for sequencing purposes.

Table 1: List of faecal samples collected

No.	Sample Name	Sample Type	Species Name	Locality
1	KKMF01	Fecal	<i>M. fascicularis</i>	Pulau Tinggi
2	KKMF02	Fecal	<i>M. fascicularis</i>	Pulau Tinggi
3	KKMF09	Fecal	<i>M. fascicularis</i>	Pulau Tinggi

Table 2: D-loop primers used (Abdul-Latiff et al., 2017)

Primer	Sequence
LATIFF1638_F	5' ACAGTCCTAGTATTAACCTGC-3'
LATIFF1689_R	5' CAAGGGGTGTTTAGTGAAGT-3'

BioEdit v7.2.3 (Hall, 1999) was used to examine the sequence chromatogram while GenBank BLASTn was used for species and sequence recognition before bioinformatic analysis was conducted using MEGA X (Kumar *et al.*, 2018). Two phylogenetic tree reconstructions were carried out: The distance-based method (Neighbor-Joining, NJ) and the character-based method (Maximum-Parsimony, MP). *M. sylvanus* was chosen as an outgroup for phylogenetic analysis (NJ and MP). Representatives of *M. fascicularis* population from different localities and subspecies are also used in the analysis, including *M. f. aureus* and *M. f. fascicularis* from Muar, Mersing, Paloh, Kota Tinggi and Pulau Tioman (Abdul-Latiff *et al.*, 2014a; 2014b).

Results and Discussion

Neighbour-Joining (NJ)

The NJ phylogenetic tree with the sum of branch length = 0.27180824 obtained is shown in Figure 1. Pulau Tinggi population (KKMF01, KKMF02

and KKMF09) and other *M. f. fascicularis* from various localities are separated from *M. f. aureus* supported with 100% bootstrap value. Within the *M. f. fascicularis* clade, the Pulau Tioman population formed a monophyletic clade, differentiated from the Johor population supported by 100% bootstrap value. All samples from the state of Johor are grouped in a poorly supported clade with only a 22% bootstrap value, indicating an inconclusive branching pattern. Even though all samples from Pulau Tinggi remain in a monophyletic clade supported by high bootstrap value (99%), the results indicate very little differentiation between the population from Pulau Tinggi and mainland Johor.

Maximum Parsimony (MP)

Generally, the MP tree (Figure 2) typically portrays a similar phylogenetic relationship as shown in the NJ tree. The 50% majority consensus parsimony tree with a consistency index of 0.673759, retention index of 0.805085 and a composite index of 0.646820 (0.542433) for all sites and parsimony-informative sites

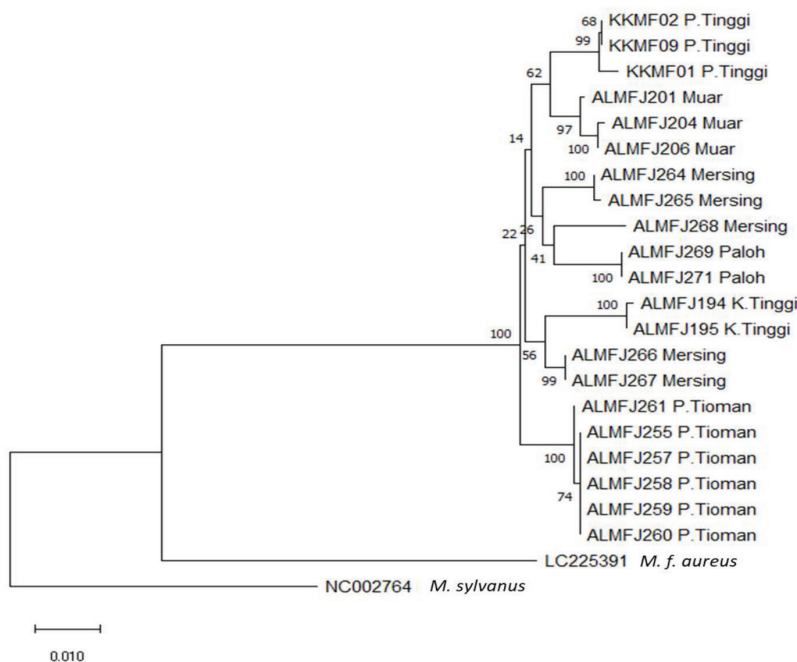


Figure 1: The neighbour-joining phylogenetic tree estimated using the Kimura-2- parameter algorithm and 1,000 bootstrap replications

(in parentheses) was obtained. While the monophyletic clade of *M. f. fascicularis* was again highly supported with 100% bootstrap value differentiated from *M. sylvanus* and *M. f. aureus*, the bootstrap support for *M. f. fascicularis* populations in Pulau Tioman, Pulau Tinggi and the state of Johor remains inconclusive.

Molecular Clock

Bayesian inference phylogeny (Figure 3) indicates 1.00 posterior probability (PP) differentiating *M. f. aureus* and *M. f. fascicularis* from *M. sylvanus*. The Pulau Tioman population was again portrayed as distinct from other populations in Johor and Pulau Tinggi with 1.00 PP, parallel with the NJ tree. Further differentiation between *M. f. fascicularis* was not proven with low PP value supporting the clades. To estimate the time of divergence for the population in Pulau Tinggi, *M. sylvanus* was used as a calibration point at ~5.5 MYA (Abdul-Latiff et al., 2014a) and indicated ~0.299 MYA for the population in Pulau Tinggi.

Previous studies have shown that *M. fascicularis* populations in Malaysia can be divided into two main clusters: The Borneo populations and Peninsular Malaysia populations (Abdul-Latiff et al., 2014a,b). In 1909, *M. f. laeta* was first proposed by Elliot (1909) which was later acknowledged by Medway (1969) and Weitzel et al. (1988) to be distributed in Pulau Tinggi. *M. f. laeta* was described as having a relatively light, grey-brown pelage with a blackish crown and tail that can be found in Pulau Tioman and Pulau Tinggi, East Coast of Malaysia. However, Abdul-Latiff et al. (2014b) have proven that the genetic population of *M. fascicularis* in Pulau Tioman cannot be classified as *M. f. laeta* but as *M. f. fascicularis* instead, according to its phylogenetic relationship. Although the results obtained from the phylogenetic analysis in this study indicate a poorly supported clade to support the differentiation of *M. f. fascicularis* clades, it surely supports the taxonomic position of the population from Pulau Tinggi as *M. f. fascicularis* and not *M. f. laeta*. However, as

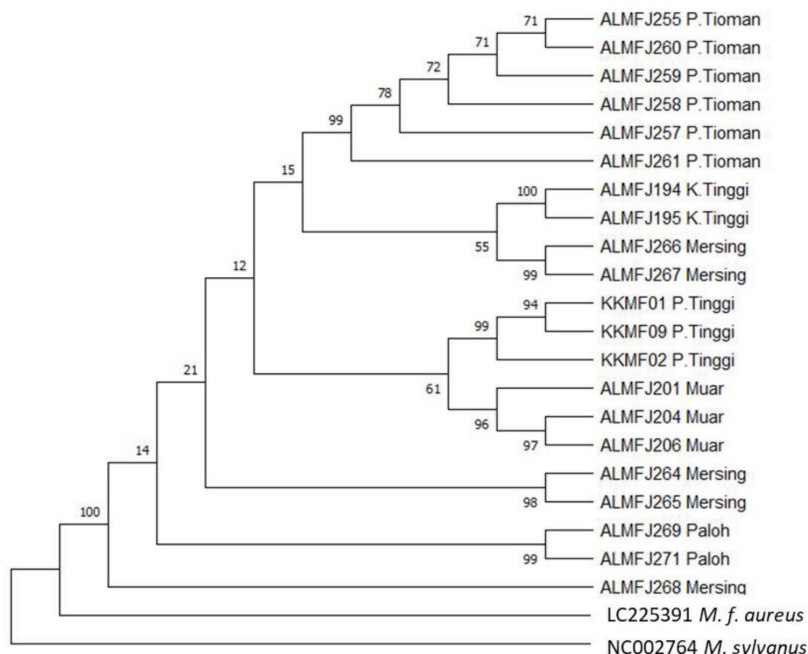
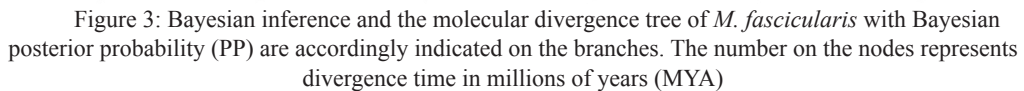


Figure 2: The maximum parsimony (MP) phylogenetic tree estimated using the TBR algorithm, heuristic searching method and 1,000 bootstrap replications



In conclusion, the research has proven that the population of long-tailed macaque in Pulau Tinggi previously assumed as *M. f. laeta* is *M. f. fascicularis*. Future studies on *M. fascicularis* at the population level are needed, especially from other localities in Pulau Tinggi, its surrounding

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