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Molecular data confirm the presence of *Nycticebus bengalensis* on Langkawi Island, Malaysia

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Abstract. *Md-Zain BM, Mohhoyua KS, Aifat NR, Ngadi E, Ayob N, Rovie-Ryan JJ, Ampeng A, Mohd-Ridwan AR, Blair ME, Abdul-Latiff MAB. 2019. Molecular data confirm the presence of* Nycticebus bengalensis *on Langkawi Island, Malaysia. Biodiversitas 20: 1115-1120.* Recent taxonomic reviews have stated the possibility of Bengal Slow Loris (*Nycticebus bengalensis*) presence in the Northern part of the Malay Peninsula. This study aims to confirm the presence of the Bengal Slow Loris in Malaysia by sequencing the mitochondrial COI gene from samples collected from Langkawi Island, Peninsular Malaysia, and Borneo. Phylogenetic analyses produced tree topologies that support the grouping of slow loris samples by their localities. The tree topologies further show that slow loris samples from Sarawak and Peninsular Malaysia form two distinct clades. The clade from Peninsular Malaysia was divided into two subclades, Langkawi and Selangor. The Langkawi slow loris subclade includes sequences from GenBank representing *N. bengalensis*, supported by a high bootstrap value. This mitochondrial DNA finding has a significant contribution to indicate the presence of the Bengal Slow Loris in Malaysia.

Keywords: Biogeography, Malaysian primates, *Nycticebus bengalensis*, *Nycticebus coucang*, phylogeny, slow loris

INTRODUCTION

The slow loris (Genus *Nycticebus*, family Lorisidae) is a nocturnal primate found in South and Southeast Asian regions (Roos et al. 2014). Species distributions range from eastern India to Indochina and southern China south to the Malay Peninsula and Java, Borneo to the western Philippines (Groves 2001; Brandon-Jones et al. 2004). There are eight species currently recognized under this genus *Nycticebus coucang*, *Nycticebus javanicus*, *Nycticebus pygmaeus*, *Nycticebus bengalensis*, *Nycticebus menagensis*, *Nycticebus kayan*, *Nycticebus bancanus* and *Nycticebus borneanus* (Munds et al. 2013; Nekaris and Starr 2015). *N. bengalensis* has the largest range of any species in the genus because it is found in Myanmar, Cambodia, southern China, northeast India, Laos, Thailand, Vietnam and Bangladesh (Brandon-Jones et al. 2004; Roos et al. 2014).

Most *Nycticebus* classifications were based on morphological data while few molecular studies have been performed (Chen et al. 2006; Cao et al. 2017). Similar morphological characteristics between species make the identification process difficult at the species or subspecies level (Blair et al. 2011). Previous molecular studies were conducted by Chen et al. (2006) and Md-Zain et al. (2009) to study the taxonomy of *Nycticebus*. Using the mitochondrial gene Cytochrome *b* (Cyt *b*), Md-Zain et al. (2009) showed separation between samples from Peninsular Malaysia and Borneo that were later considered as different species (Roos et al. 2014). In addition, Chen et al. (2006) found that *N. c. coucang* and *N. bengalensis* could not be distinguished, probably due to the limited geographic sampling of this study or misidentification of specimens. While eight species are currently recognized in the genus (Nekaris & Starr 2015), many more molecular systematic studies need to be carried out to improve our understanding of *Nycticebus* genetic identity and current distribution.

Previously, only *N. coucang* has been described as distributed in the Malay Peninsula, with several subspecies (Brandon-Jones et al. 2004). Recently, Rovie-Ryan et al. (2018) rediscovered *N. c. insularis* in Tioman Island using two mitochondrial loci, the Cyt *b* and D-loop region. The presence of *N. bengalensis* has been hypothesized in the Northern part of the Malay Peninsula; however, no scientific evidence is available to confirm the presence. This paper presents the first-ever molecular scientific study to confirm the presence of *N. bengalensis* in Malaysia using the Cytochrome Oxidase I (COI) mitochondrial region as a candidate locus. The COI gene has been widely used in systematic, population and phylogeography studies in primates (Abdul-Latiff et al. 2017) and other mammals

(Bakar et al. 2017, 2018; Syed-Shabthar et al. 2013; Rosli et al. 2011). The COI gene has also been selected as a good candidate marker for DNA barcoding for species identification and wildlife forensic applications (Md-Zain et al. 2018a,b; Mohd-Yusof et al. 2018).

MATERIALS AND METHODS

DNA extraction, Polymerase Chain Reaction (PCR) and sequencing

Fecal, tissue and FTA cards of *Nycticebus* were collected from Sarawak, Selangor and Langkawi Island comprising a total of 10 genetic samples (Table 1). Tissue samples were collected from road kill specimens while other genetic samples were provided by the Department of Wildlife and National Park (PERHILITAN) Peninsular Malaysia and Sarawak Forestry Department with special research permit by Ministry of Natural Resources and Environment (NRE 600-2/2/21 Jilid 6 (35). In this study, three different extraction kits were used: the innuPREP Stool DNA Kit (Analytik Jena, Germany) for fecal samples, innuPREP DNA Mini Kit (Analytik Jena, Germany) for tissue samples and innuPREP Forensic Kit (Analytik Jena, Germany) for FTA cards. DNA was extracted from 0.5-1.0g of fecal samples (Abdul-Latiff et al. 2014a, b), 0.02g of tissue samples (Aifat et al. 2016a) and a few punches of FTA cards following the manufacturer's protocol.

We used representatives of *N. menagensis*, *N. bengalensis* and *N. pygmaeus* sequences to conduct a comparative analysis of genus *Nycticebus*. Reference sequences for these taxa are available in GenBank for the Cytochrome Oxidase I (COI) region. Table 2 shows a primer sequence for the COI region designed specifically for *Nycticebus* (Blair, Unpublished) that was used to amplify all the in-hand genetic samples.

A total of ~ 400 bp fragment of the mitochondrial COI region were successfully amplified through polymerase chain reaction (PCR) using Mastermx MyTaq Red Mix (Bioline) and Mastercycler Nexus (Eppendorf North America, Inc.). The PCR involved a three-step PCR protocol following Abdul-Latiff et al. (2017) (Table 3 and Table 4). Purification was done by using doublePURE kit, and the samples were subsequently sent to Apical Scientific Sdn Bhd (Malaysia) for sequencing purposes.

Table 2. Primer sequence of COI region

Primer	Sequence (5'-3')	Reference
5288F	CACCTCGAGGCCTGGTAAAAA GGG	Blair, Unpublished
5704R	GCCGGCTCCGGCCTCAACTA	Blair, Unpublished

Table 3. PCR cocktail involved in DNA amplification

Components	Final concentration	Volume (µL)
My Taq Red Mix		12.5
Forward primer	20 µmol	1.0
Reverse primer	20 µmol	1.0
DNA template		3.0
ddH_2O		7.5
Total		25

Table 4. PCR profile

Parameter	Temperature (°C)	Time	Cycle
Pre-denaturation	95	3 minutes	1
Denaturation	95	15 seconds	30
Annealing	60.8	30 seconds	30
Elongation	72	10 seconds	30
Final elongation	72	10 minutes	1

Table 1. The list of samples used in this study

Sample name	Types of samples	Locality	GenBank accession no.
LANGKAWI 1	FTA card	Langkawi Island	
LANGKAWI 2	Tissue	Langkawi Island	
LANGKAWI 3	Tissue	Langkawi Island	
N. coucang A024	FTA card	Selangor	
N. coucang ZZ098	FTA card	Selangor	
N. coucang CS	FTA card	Selangor	
N. coucang C4	FTA card	Selangor	
N. coucang C7	FTA card	Selangor	
N. coucang SELANGOR	FTA card	Selangor	
N. menagensis BMNCQ 21	Fecal	Lundu, Sarawak	
N. menagensis	GenBank		GQ259901 (Somura et al. 2012)
N. bengalensis	GenBank		NC021958 (Finstermeier et al. 2013)
N. bengalensis	GenBank		KC977312 (Somura et al. 2013)
N. bengalensis	GenBank		KC757405 (Finstermeier et al. 2013)
N. pygmaeus	GenBank		NC033381 (Ni et al. 2016)
N. pygmaeus	GenBank		KX397281 (Ni et al. 2016)
N. pygmaeus	GenBank		GQ259902 (Somura et al. 2012)
Loris tardigradus	GenBank		NC012763 (Matsui et al. 2009)

Sequence and phylogenetic analysis

Bioedit Sequence Alignment Editor was used to edit the obtained raw sequences and blasted through GenBank BLASTn for sequence similarity searches analysis (Aifat et al. 2016b; Abdul-Latiff et al. 2019). All the sequences were aligned using MEGA7 ClustalW multiple alignments (Kumar et al. 2016). Two levels of analysis were performed namely sequence and phylogenetic analysis. MEGA7 and PAUP 4.0B10 (Swofford 2002) software were used in sequence and phylogenetic analyses to reconstruct phylogenetic trees and genetic distances. Phylogenetic trees were constructed using character-based (maximum parsimony (MP) and distance-based (neighbor-joining (NJ) methods. The tree bisection and reconnection (TBR) algorithms were used for the MP tree. The heuristic searching method and 1000 random stepwise additions were applied to find the best tree through the application of the 50% consensus majority rule. All the trees constructed underwent 1000 bootstrap replications to obtain the bootstrap confidence level. The Kimura 2-parameter model was used in NJ tree reconstructions tested with a bootstrap value of 1000.

RESULTS AND DISCUSSION

COI sequence analysis

Sequence analysis

A total of 404 bp of the COI gene for ten slow loris samples were successfully sequenced and used in the final alignment. The sequences obtained were blasted against NCBI's GenBank database for species identification (Table 5). Out of the total 404 bp, 272 (67.33%) are conserved sites, 66 (16.3%) variable sites, 54 (13.36%) parsimony uninformative sites and 12 (2.97%) parsimony informative sites. The mean nucleotide frequencies were, for thymine (T) = 30.5%, cytosine (C) = 21.6%, adenosine (A) = 26.6%, and guanine (G) = 19.6%. The observed genetic distance ranged from 0-21.3% (Table 6). The highest genetic distance value is 21.3%, between *N. pygmaeus* and the outgroup (*Loris tardigradus*). Samples from Selangor showed the lowest genetic distances (A024, CS, ZZ098, C4, and C7) with 0.0% indicating that the individuals within this population are genetically the same.

Phylogenetic trees

Information gained from the reconstruction of the most parsimonious tree (length: 86) using the MP method are shown with a consistency index (0.7959), retention index (0.8718) and composite index (0.6939). NJ and MP trees produced two major groups separating slow loris samples from Sarawak (*N. menagensis* and BMNCQ21) from those from Peninsular Malaysia with more than a 55% confidence value (Figure 1 and Figure 2). Support for tree topologies was analyzed through NJ bootstrap estimates with 1000 replicates. The trees grouped all the individuals from the Selangor in the same clade with a bootstrap confidence value of more than 60% (C7, C4, ZZ098, A024, and CS). Individuals from Langkawi and Thailand (*N. bengalensis*) are grouped in the same clade suggesting that the individuals are the same species, consistent with our GenBank BLAST analysis. The separation between *N. bengalensis* and *N. coucang* clades was supported by high bootstrap value (99%).

The phylogenetic clade formation shown in this study is consistent with previous studies conducted on the molecular phylogeny of *Nycticebus*. While this study did not employ the real genetic samples of *N. pygmaeus* as others have done (Chen et al. 2006; Somura et al. 2012), we managed to produce the same tree topology in which *N. pygmaeus* diverged earlier as compared to *N. bengalensis* and *N. coucang* (Rovie-Ryan et al. 2018). We also find support for following suggestions by Rovie-Ryan et al. (2018) to utilize samples from known and credible localities only, thus increasing the confidence level of our analysis confirming the presence of *N. bengalensis* in Langkawi Island. We are also aware of the notion put forth by Groves (2001) that there is a possibility of a hybridization event occurring between *N. coucang* and *N. bengalensis* near the Isthmus of Kra; however, our phylogenetic analysis shows a clear distinction between the species. Although our geographic sampling is the most thorough to date for slow lorises in Malaysia, hybridization could still be supported by a higher resolution of geographic sampling in the Isthmus.

Table 5. The results of the species confirmation using the GenBank BLAST

Sample	Marker	E	% Identity percentage (%)	Species	GenBank Accession No.
A024	*COI*	0	99	*coucang*	AJ309867.1
CS	*COI*	0	99	*N. coucang*	AJ309867.1
C4	*COI*	0	99	*N. coucang*	AJ309867.1
C7	*COI*	0	99	*N. coucang*	AJ309867.1
SELANGOR	*COI*	0	99	*N. coucang*	AJ309867.1
BMNCQ 20	*COI*	0	99	*N. coucang*	AJ309867.1
Langkawi 1	*COI*	0	100	*N. bengalensis*	KC977312.1
Langkawi 2	*COI*	0	100	*N. bengalensis*	KY436589.1
Langkawi 3	*COI*	0	100	*N. bengalensis*	KY436589.1
ZZ098	*COI*	0	99	*N. coucang*	AJ309867.1

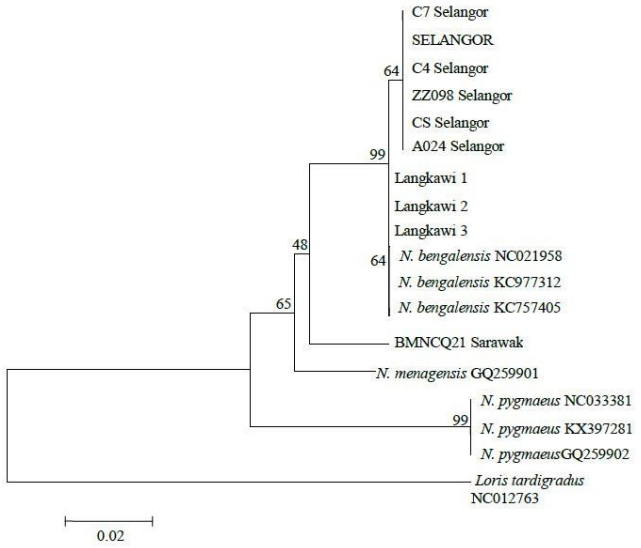

Figure 1. Neighbor-Joining (NJ) tree of slow loris samples based on 404 bp of the mtDNA COI gene. Values shown next to branches are the bootstrap estimates with 1000 replicates

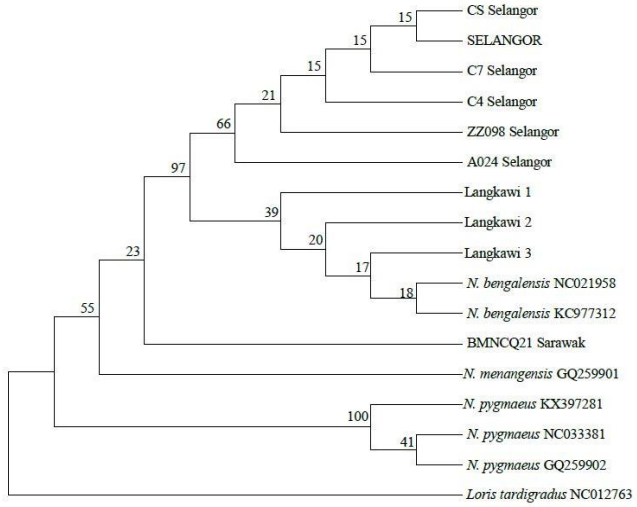

Figure 2. Maximum Parsimony (MP) tree of slow loris samples based on 404 bp of the mtDNA COI gene. Values shown above the branches are bootstrap estimates with 1000 replicates

The argument that *N. bengalensis* might be present in northern Peninsular Malaysia is not new (Groves 2001; Roos et al. 2014). In that region, the southernmost confirmed distribution as acknowledged by Streicher et al. (2008) is near southwestern Satun, Thailand and the direct distance from this area to Langkawi Island is less than 30 KM (by sea). We may also consider that *N. bengalensis* might be present in northern Perlis, surviving in the remnants of Perlis State Park, the distance between these two areas is less than 40 KM. Thus, we hypothesize that the populations of *N. bengalensis* on Langkawi Island are natural populations not recorded by previous studies. This is not surprising as this understudied group of primates has received less attention as compared to other species (Blair et al. 2011), further supported by the rediscovery of *N. c. insularis* in Tioman Island, Malaysia by Rovie-Ryan et al. (2018). This attention-deprived situation can largely be attributed to two factors as highlighted by Nekaris et al. (2008): *Nycticebus* species are abundant in some areas but genuinely rare in others, or it takes time for surveyors to learn how to survey nocturnal lorises accurately, or alternatively, it takes time for the lorises to adapt to the presence of surveyors.

The findings of this research have significant conservation implications for the slow loris in Malaysia, in addition to significant findings for slow loris biogeography. Slow lorises are among the most commonly traded protected primates in marketplaces across their ranges (Nekaris & Nijman 2007) and have long been exploited in traditional medicines, with reports dating back to 1900 (Ridley 1900). Throughout Asian countries, due to superstitious belief, slow lorises are considered to cure up to 100 ailments; thus they are heavily hunted and killed for their perceived medicinal value (Starr et al. 2010; Thach et al. 2018). The Department of Wildlife and National Parks Malaysia (PERHILITAN) are actively combatting slow loris pet trade issues, and all confiscated animals will be temporarily placed in the National Wildlife Rescue Centre (NWRC) for assessment before releasing them to their natural environment. Thus, through this research, PERHILITAN will now be able to safeguard the unique gene pool of *N. bengalensis* in Langkawi Island and *N. coucang* in mainland Peninsular Malaysia by using molecular and analysis to identify the confiscated slow lorises prior to the release of healthy individuals. Nur-Syuhada et al. (2016) have also reported a translocation of *N. coucang* from Hulu Terengganu, Terengganu Malaysia due to Hulu Terengganu Hydroelectric Project in the area. Findings in this research should well be incorporated to any management and conservation action plan in Malaysia to avoid translocating *N. coucang* and *N. bengalensis* to the wrong habitat by assuming all slow lorises in Malaysia belong to *N. coucang*.

MtDNA data has successfully identified the clear distinction of *N. bengalensis* from Langkawi Island as compared to *N. coucang* only by utilizing the COI region. Although this region is conserved (Md-Zain et al. 2018a), it has proven to be effective locus to identify different species of slow loris. Rovie-Ryan et al. (2018) reported an unresolved topology of a phylogenetic tree of *Nycticebus* based on the Cytochrome *b* region, although the data are not shown. For future study, we suggest the use of other loci which have been proven to resolve species-level phylogeny in primates such as the D-*loop* (Abdul-Latiff et al. 2014b), *ND4* (Takacs et al. 2005) and also a reattempt of Cyt *b* using different, species-specific primers as compared to Rovie-Ryan et al. (2018), as the vast data of Cyt *b* on GenBank is useful for comparative analysis purposes, especially in biogeography (Abdul-Latiff et al. 2017; Abdul-Latiff et al. 2019). Future hybrid confirmation will also need to be investigated between *N. coucang* and *N. bengalensis.*

Table 6. Genetic distance percentage (%) between individual based on COI sequence

	Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*N. coucang* A024_Selangor																		
2	*N. coucang* CS_Selangor	0.0																	
3	*N. coucang* ZZ098_Selangor	0.0	0.0																
4	*N. coucang* C4_Selangor	0.0	0.0	0.0															
5	*N. coucang* C7_Selangor	0.0	0.0	0.0	0.0														
6	*N. coucang* SELANGOR	0.0	0.0	0.0	0.0	0.0													
7	Langkawi 1	0.3	0.3	0.3	0.3	0.3	0.3												
8	Langkawi 2	0.3	0.3	0.3	0.3	0.3	0.3	0.0											
9	Langkawi 3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.0										
10	*N. bengalensis* NC021958	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.0	0.0									
11	*N. bengalensis* KC977312	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.0	0.0	0.0								
12	*N. bengalensis* KC757405	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.0	0.0	0.0	0.0							
13	*N. pygmaeus* NC033381	8.7	8.7	8.7	8.7	8.7	8.7	8.3	8.3	8.3	8.3	8.3	8.3						
14	*N. pygmaeus* KX397281	8.7	8.7	8.7	8.7	8.7	8.7	8.3	8.3	8.3	8.3	8.3	8.3	0.0					
15	*N. pygmaeus* GQ259902	8.7	8.7	8.7	8.7	8.7	8.7	8.3	8.3	8.3	8.3	8.3	8.3	0.0	0.0				
16	*N. menagensis*	4.3	4.3	4.3	4.3	4.3	4.3	4.0	4.0	4.0	4.0	4.0	4.0	7.5	7.5	7.5			
17	BMNCQ21 Sarawak	4.0	4.0	4.0	4.0	4.0	4.0	3.6	3.6	3.6	3.6	3.6	3.6	7.6	7.6	7.6	4.3		
18	LORIS	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	21.3	21.3	21.3	19.5	19.5	

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