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MOLECULAR SYSTEMATIC POSITION OF THE SARAWAK MALAY BADGER, *Mydaus javanensis*

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ABSTRACT

The presence of the Malay badger, *Mydaus javanensis*, has been recorded for nearly 100 years in Sarawak, Malaysia, but it has rarely been seen. In addition, this animal is among the least studied carnivores in Borneo. The Malay badger is not protected under the Sarawak Wildlife Protection Ordinance. To initiate conservation efforts, we conducted a phylogenetic analysis to characterize the Malay badger's genetic attributes. In August 2013, a Malay badger was trapped at Mentung Berawan, Serian, and sent to the Matang Wildlife Centre. We managed to collect its genetic materials and sequenced 356 bp of 12S rRNA and 405 bp of cytochrome *b* (Cyt *b*) genes. We portrayed its phylogenetic relationships with other Mephitidae family members and calculated its molecular divergence. Our results indicated that the Malay badger could be distinguished from its sister taxon, *M. marchei*. The teledu clade diverged 2.71 million years ago, after the divergences of *Mephitis mephitis* and *Spilogale putorius*.

Key words: Malay Badger, *Mydaus javanensis*, Teledu, Malayan Stink Badger, Mephitidae

INTRODUCTION

The Malay badger, which has the scientific name *Mydaus javanensis*, is also known as the teledu, Sunda stink badger, Malayan stink badger, or Indonesian stink badger; it can be found on Borneo, Sumatra, and Natuna Island (Wilson & Reeder, 2005). This carnivore is common to lower elevations (Payne & Francis, 1985), but it has also been found at higher elevations on the Kelabit Plateau in Sarawak (Lawrence, 1939). The IUCN Red List has categorized the Malay badger under Least Concern; thus, more ecological surveys are warranted for determining the species' correct conservation status.

M. javanensis and *M. marchei* are the only two species categorized under the genus *Mydaus*, family Mephitidae (Dragoo & Honeycutt, 1997). *M. marchei*, the Palawan stink badger – the sister taxon to the Malay badger – lives in the Palawan and Calamian Islands, Philippines (Nowak, 2005). These are the only two stink badgers in existence (Nowak, 2005), and the only skunks that can be found outside the Americas (Dragoo & Honeycutt, 1997). *M. javanensis* can be distinguished from *M. marchei* by its smaller teeth, longer tail, larger ears, white crown, and white stripe extending to the tail (Figure 1: Long & Killingley, 1983). Three subspecies are recognized, namely *M. j. javanensis* Desmarest, *M. j. lucifer* Thomas, and *M. j. ollula* Thomas (Strien, 1986).

Based on the morphological and fossil evidence, the genus *Mydaus* was originally grouped with the family Mustelidae (Wozencraft, 1993). However, genetic analyses suggested that *Mydaus* should be placed with skunks (genera *Conepatus*, *Mephitis*, *Spilogale*) in a separate family, the Mephitidae

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Fig. 1. Phenotypic appearance of Sarawak *M. javanensis*

(Dragoo and Honeycutt, 1997). Few studies are available to portray carnivores' phylogenetic relationships by including *Mydaus* as one of the taxa studied. However, the number of samples is limited, with few mitochondrial DNA (mtDNA) loci having been sequenced. Therefore, in this study, we include two mtDNA loci to determine the phylogenetic position of *M. javanensis* from Sarawak, Malaysia. Mitochondrial DNA (mtDNA) has been widely used to infer phylogenetic relationships of closely related in vertebrates due to its unique characteristics (Ang *et al*., 2012; Md-Zain *et al*., 2010; Rosli *et al*., 2014; Md-Zain *et al*., 2018; Abdul-Latiff *et al*., 2019).

MATERIALS AND METHODS

DNA extraction, amplification, and sequencing

The Malay badger's fecal sample was collected in a 95% ethanol vial. Total genomic DNA was extracted by using the standard extraction kit and protocol provided by innuPREP Stool DNA Kit (Analytik Jena) (Aifat *et al*., 2016). Polymerase chain reaction (PCR) was performed using a 25 µl reaction mixture containing 1 µl of genomic DNA, 2.5 µl of PCR buffer 10X, 1 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 1.5 µl each of 10 pmol/µl primer and 4 units of *Taq* DNA Polymerase in PTC-100 Thermal Cycler (MJ Research Inc.). The partial 12S rRNA gene fragment of approximately 450 bp was amplified using the universal mammal forward primer L1091 (52-CTG GGA TTA GAT ACC CCA CTAT-32) and reverse primer H1478 (52-GAG GGT GAC GGG CGG TGT GT-32; Kocher *et al.,* 1989). The cytochrome *b* (Cyt *b*) gene primers for amplifying 1,100 bp were R-L14724 (52-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-32; Kocher *et al*., 1989) and UH15155 (52-GGA ATT CAT CTC TCC CGG TTT ACA AGA C-32; Irwin *et al*., 1991). The PCR conditions for the genes were as follows: 4 minutes of denaturation at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 58°C (12S rRNA) and 55°C (Cyt *b*), 1 minute at 72°C, and a final 7-minute extension at 72°C, before cooling to 4°C for 10 minutes. The DNA from PCR products was purified using the Vivantis G-F1 PCR Clean-up Kit and sent directly to the sequencing service company, First Base Sdn. Bhd. (Malaysia), for sequencing.

Phylogenetic analysis

The sequencing result was exported as a FASTA sequence file (Abdul-Latiff *et al*., 2014a). The 12S rRNA and Cyt *b* sequences were aligned separately using the ClustalW multiple alignment algorithm of BioEdit (Ang *et al*., 2011) together with sequences obtained from GenBank. All sequences were analyzed using MEGA 5.0 and PAUP 4.0b10 for phylogeny reconstruction (Swofford, 2002). Genetic distance and sequence polymorphism were analyzed using MEGA 5.0. The two methods of analysis in PAUP included the following: (1) neighbor joining (NJ) with Kimura's 2-parameter model, which considered the unequal rates of evolution in terms of transition and transversion, but at the same time, assumes an equal distribution of nucleotide composition, and (2) maximum parsimony (MP) with stepwise addition of 1,000 replicates in a heuristic search and a 50% majority rule consensus. In MP, gaps are treated as missing data, and transitions and transversions have equal weight; the heuristic search is carried out with a Tree Bisection and Reconnection branch-swapping algorithm (Syed-Shabthar *et al*., 2013). In this study, all the trees were subjected to bootstrap analysis with 1,000 replicates to obtain bootstrap value support.

Bayesian and molecular clock

The most suitable model for both Cyt *b* and 12S rRNA of mtDNA was selected using the Modeltest ver. 3.7 software by means of the Akaike information criterion (AIC) requirements. The HKY+I+G model with gamma shape parameters of 1.4067 and 1.0992, respectively, were selected for the two loci. Metropolis-coupled Markov chain Monte Carlo (MCMC) was run with 10 million generations, and the tree was sampled every 1,000 generations. Ten percent of the trees obtained were discarded as burn-in, and the majority consensus rule was employed for the remaining trees. Posterior probabilities (PP) were summarized on each branch.

Molecular clock analysis was carried out to determine the divergence date of *Mydaus javanensis*. To root the ingroup datasets to a monophyletic state, the outgroup datasets contained only *Arctocephalus townsendi*. *Mephitis* datasets were created to be used as the most recent common ancestor, as the earliest fossil evidence of the genus *Mephitis* is from the early Pleistocene carbon dating ~1.8 million years ago (MYA) originated from the Broadwater site in Nebraska (Kúrten & Anderson, 1980). The uncorrelated lognormal relaxed-clock model with uniform priors on the mean (0.100) and standard deviation (0.10) was used to estimate the substitution rate for all nodes in the tree. The birth-death speciation model (Gernhard, 2008), which suggests that births and deaths of lineages occur at a constant rate and are independent, was also used to reconstruct the starting tree. MCMC was run for 10 million generations, and the trees were sampled every 1000 generations, with 1% of the sample discarded as burn-in. The maximum-clade-credibility tree topologies were calculated using posterior distribution, and TreeAnnotator ver. 1.7.5 was employed to produce the final summary trees (Abdul-Latiff *et al*., 2014a).

RESULTS

Sequence polymorphism and genetic distance

Amplification products of 356 and 405 bp were obtained for the 12S rRNA and Cyt *b* fragments, respectively. The sequence analysis indicated that, of the 95 variable sites in the 12S rRNA, 66 were parsimony informative. Meanwhile, among the 165 variable sites in the Cyt *b* gene, 139 were found to be parsimony informative. There were 29 and 26 single-nucleotide polymorphisms for 12S rRNA and Cyt *b*, respectively. The Ti/Tv ratio of 12S rRNA was 2.6, while it was 2.7 for Cyt *b*. The pairwise genetic distances of 12S rRNA and Cyt *b* were calculated using the Kimura two-parameter model (Table 1 and Table 2), focusing on the family Mephitidae (*Mydaus*, *Mephitis*, and *Spilogale*). The genetic distance of the teledu samples and species from the genus *Mydaus* showed a minimum genetic distance of 0.026 and 0.024 for 12S rRNA and Cyt *b*, respectively. Meanwhile, maximum genetic distances of 0.113 and 0.255 for 12S rRNA and Cyt *b* were showed between teledu and the genus *Spilogale.*

Table 1. Genetic distance of Family Mephitidae based on 12S rRNA sequences

	1	2	3	4	5	6	7	8
1. Teledu	–							
2. *Mydaus marchei*_U78342	0.026	–						
3. *M. mephitis*_U78338	0.103	0.106	–					
4. *M. mephitis*_Y08517	0.106	0.103	0.014	–				
5. *M. mephitis*_NC020648	0.106	0.103	0.014	0.000	–			
6. *S. putorius*_NC010497	0.113	0.103	0.068	0.059	0.059	–		
7. *S. putorius*_U78346	0.103	0.096	0.059	0.056	0.056	0.032	–	
8. *S. putorius*_Y08518	0.113	0.103	0.068	0.059	0.059	0.000	0.032	–

Table 2. Genetic distance of Family Mephitidae based on Cyt *b* sequences

	1	2	3	4	5	6	7
1. Teledu	–						
2. *Mydaus javanensis*_AB564095	0.024	–					
3. *M. mephitis*_NC0202648	0.240	0.241	–				
4. *M. mephitis*_AJ536014	0.240	0.241	0.008	–			
5. *M. mephitis*_JN008709	0.252	0.252	0.036	0.028	–		
6. *S. putorius*_X94928	0.255	0.255	0.149	0.149	0.134	–	
7. *S. putorius*_AJ536015	0.255	0.255	0.149	0.149	0.134	0.000	–

Phylogenetic trees

The teledu's phylogenetic position is shown in Figure 2 (12S rRNA) and Figure 3 (Cyt *b*). The NJ trees exhibited high similarity to the MP and Bayesian trees. Two main clades were formed in the superfamily Musteloidea for the 12S rRNA gene. Clade A consisted of the family Mephitidae, while clade B consisted of the family Mustelidae. The Mephitidae and Mustelidae families formed monophyletic clades, supported by bootstrap values of 98 and 69, respectively. Mephitidae were further separated into two clades: Teledus and *Mydaus marchei* formed one clade, while *Mephitis mephitis* and *Spilogale putorius* formed the other. For the molecular clock, using calibration points of *Mephitis mephitis*, it was found that the teledu clade diverged 2.7061 MYA, after *Mephitis mephitis* and *Spilogale putorius*. In addition, the Cyt *b* tree showed that two main clades formed the same topology as for 12S rRNA. Clade A consisted of the family Mustelidae, and clade B consisted of the family Mephitidae; this was supported by bootstrap values of 94 and 97, respectively. Teledus and *Mydaus javanensis* formed a clade, with a bootstrap value of 100, to become a sister clade to a clade comprising the *Mephitis* and *Spilogale* genera.

DISCUSSION

The results of the present study of 12S rRNA and Cyt *b* genes provide evidence to clarify the classification of teledu. From the phylogenetic tree, in different loci, different species were used to clarify the phylogenetic position of teledu. In 12S rRNA, *Mydaus marchei* was used in the

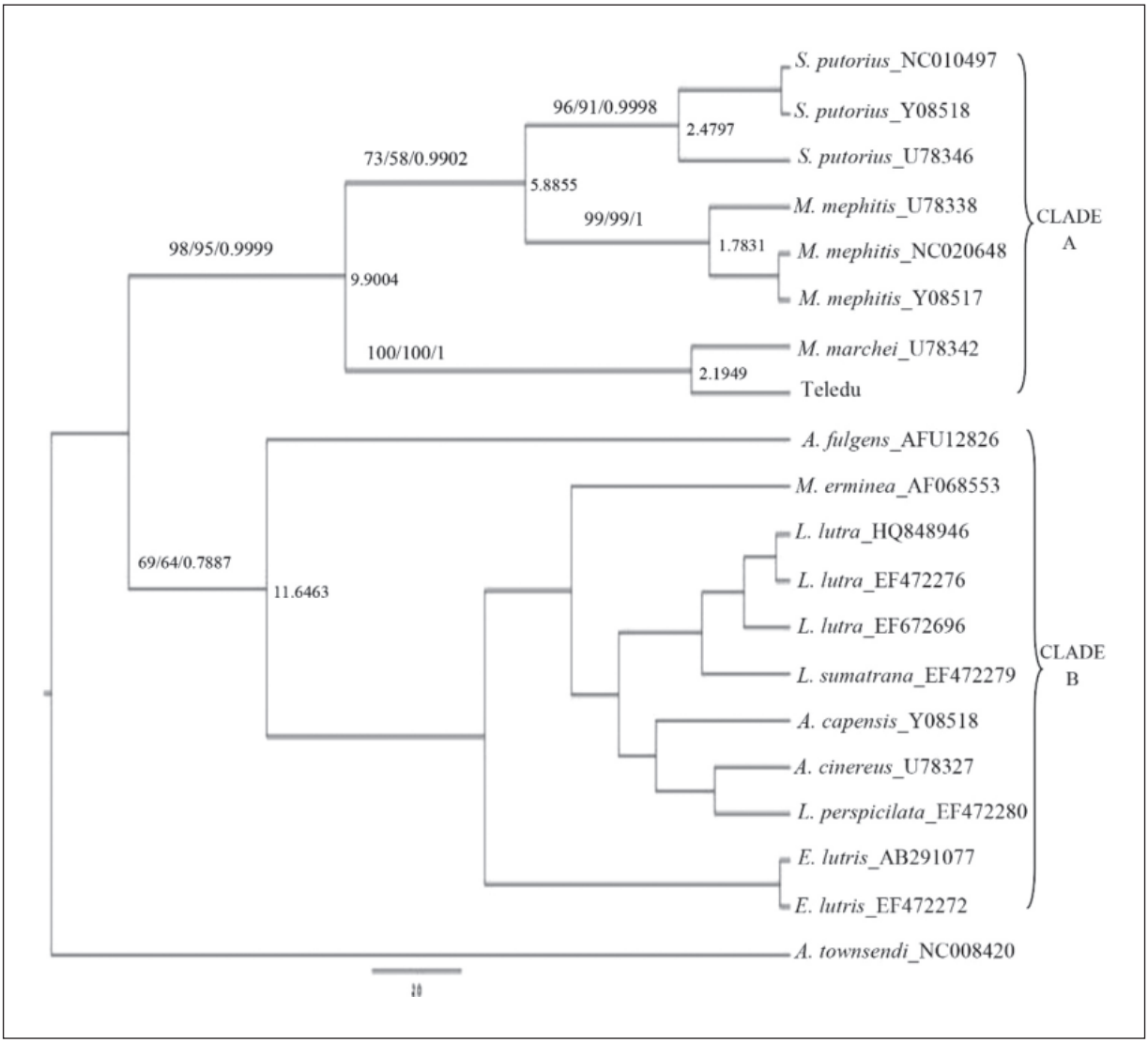

Fig. 2. 12S rRNA phylogram tree of teledu. Branch lengths are drawn according to those estimated by the NJ algorithm with numbers on nodes indicating support values (first:NJ, second: MP, third: Bayesian inference, the value between the nodes of the branch: divergence time).

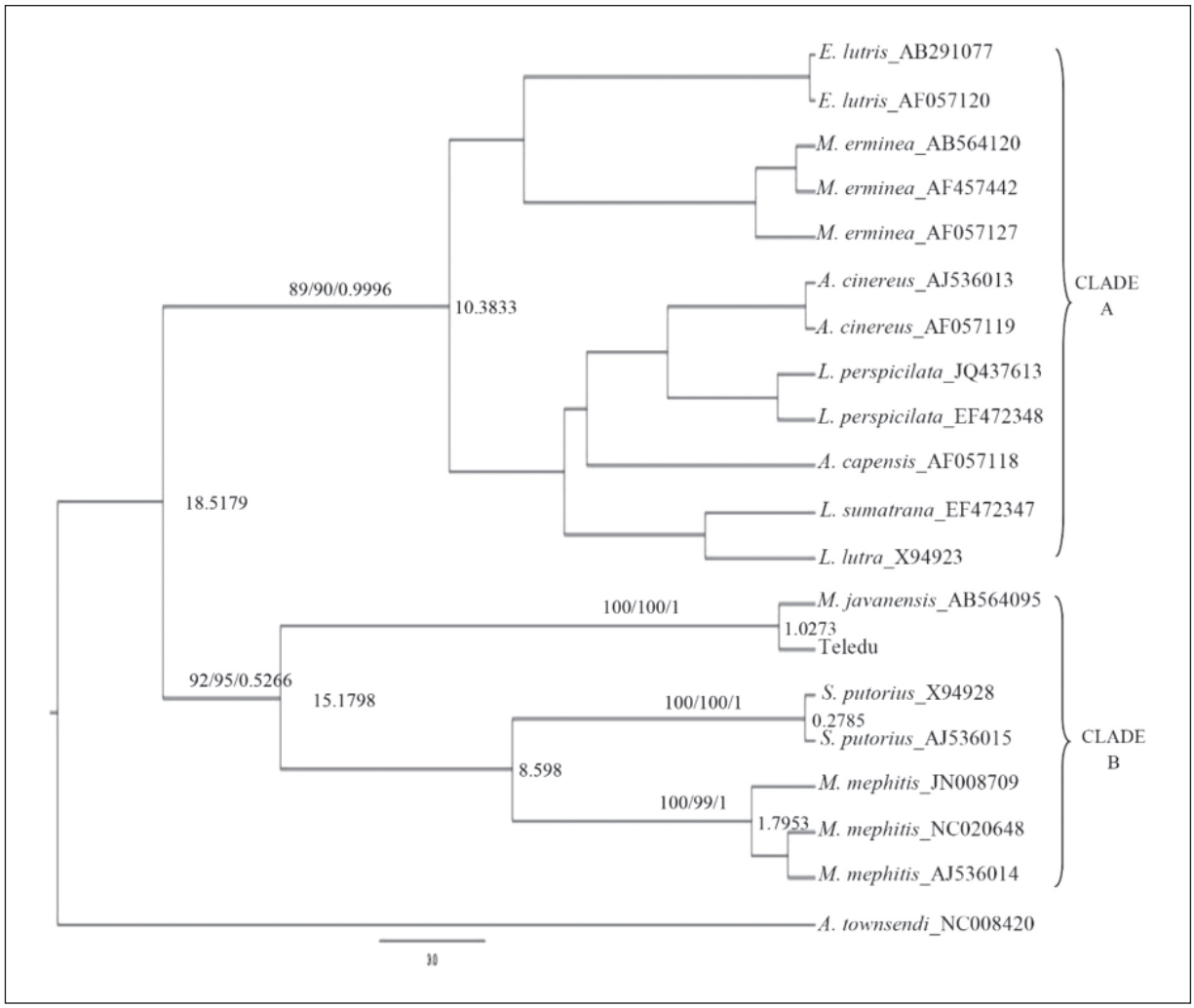

Fig. 3. Cyt *b* phylogram tree of Teledu. Branch lengths are drawn according to those estimated by the NJ algorithm with numbers on nodes indicating support values (first:NJ, second: MP, third: Bayesian inference, the value between the nodes of the branch: divergence time).

phylogenetic tree, while *Mydaus javanensis* was used in the Cyt *b* phylogenetic tree. This is because there is no sequence in the gene bank database for both species needed to be used in this analysis. Either one has the 12S rRNA sequence and not the Cyt *b* locus, or vice versa.

The genus *Mydaus* was used for this analysis because it is thought that the teledu is one of the species in the family Mephitidae and nearest to the genus *Mydaus*. Traditionally, *Mydaus* was placed with the Mustelidae based on morphological data and fossil evidence (Wozencraft, 1993); however, numerous authors placed stink badgers close to skunks based on recent genetic analyses, suggesting that stink badgers should be placed in Mephitidae along with skunks (*Conepatus*, *Mephitis*, and *Spilogale*). From the molecular features, both the mitochondrial and nuclear sequences have consistently demonstrated that skunks and stink badgers (*Mydaus*) descend from a common ancestor, and together, form a lineage with Mephitidae. Thus, they diverged prior to the split between Mustelidae and Procyonidae (Dragoo & Honeycutt, 1997). In addition, the teledu is one of the indigenous common names for *M. javanensis* (Corbet & Hill, 1992). With this evidence, the phylogenetic tree generated from the analysis of the NJ, MP, Bayesian inference, and molecular clock showed that teledus and the genus *Mydaus* form one clade, supported by a bootstrap value of 100, which is considered a higher value.

As shown in the analyses of 12S rRNA and Cyt *b*, the genus *Mydaus* clade forms a paraphyletic clade with other individuals in the family Mephitidae, namely *Mephitis mephitis* and *Spilogale putorius*. Furthermore, since the teledu sample was from Sarawak, it was proven that the teledu is *M. javanensis*, as one study stated that *M. javanensis* is distributed in the area where this sample was found. This automatically showed

that, based on its molecular characteristics, the genus *Mydaus* is in the family Mephitidae instead of Mustelidae, where it was previously placed based on morphological data.

The other species in genus *Mydaus* is *M. marchei*, also known as the Palawan stink badger. *M. marchei* is the only congener of *M. javanensis* found on the Busuanga, Calauit, and Palawan Islands in the Philippines (Heaney, 1998). These two species are the only species of skunks (family Mephitidae) outside the Americas (Dragoo & Honeycutt, 1997). For this reason, the teledu form into one clade with *M. marchei* in the analyses of 12S rRNA, as the genus is the same, although the distribution of *M. marchei* is not in the range of the teledu.

Molecular clock analysis was performed to estimate the molecular divergence date of teledu in the family Mephitidae. This date was estimated by calibrating it against independent evidence from fossil record for the species *M. mephitis*. Calibration was needed because the molecular clock cannot assign a concrete date; therefore, the fossil record is needed as evidence for dates. The fossil record of *M. mephitis* was recorded about 1.8 MYA (Barton & Wisely, 2012). The pattern of teledu molecular evolution was estimated by sequences amplified from mtDNA 12S rRNA and Cyt *b*. From the analysis, the teledu was diverged 2.19 MYA (12S rRNA) and 1.03 MYA (Cyt *b*), after *M. mephitis*. 12S rRNA and Cyt *b* exhibited differential divergences dates of teledu in the family Mephitidae. This is because the 12S rRNA sequence exhibited less variable sites than Cyt *b*, with 95 and 165 sites, respectively. A comparison of sequence variation in Cyt *b* and 12S rRNA revealed dissimilar rates of mutation (Masuda *et al.,* 1996). The slower rate of mutation in 12S rRNA is consistent with reconstructions across mammalian orders (Gemmell & Westerman, 1994), as well as within Carnivora (Zhang & Ryder, 1993).

This study employed two loci of mitochondrial DNA, namely 12S rRNA and Cyt *b*. 12S rRNA is widely used for working out the evolutionary relationships among organisms. Furthermore, this locus has an ancient origin, and it can be found in all known forms of life. This ribosomal RNA is one of only a few gene products present in all cells (Smit *et al*., 2007). Thus, genes that encode the rRNA or rDNA are sequenced to identify organisms' taxonomic groups, calculated related groups, and estimate rates of species divergence. Based on previous research, using 12S rRNA, Flynn *et al*. (2005) recovered all the higher level Carnivora clades that had been robustly supported by the analyses using morphological and molecular data. In addition, the study further determined the relative positions of the major lineages within Carnivora. For the lower level of taxa, representing the superfamily level, Dragoo and Honeycutt (1997) studied the phylogenetic relationships of skunks to Mustelidae and other caniform carnivores by using mitochondrial DNA (mtDNA) sequence data from 12S rRNA and 16S rRNA. In this study, they found that the Mustelidae form a paraphyletic group with the skunks (genera *Conepatus*, *Mephitis*, and *Spirogale*) and Oriental stink badger (*Mydaus*), forming a monophyletic clade that is separate from a clade containing the individuals from Mustelidae.

Due to its sequence variability, Cyt *b* is commonly used as a region of mitochondrial DNA to determine the phylogenetic relationships between organisms. In addition, it is widely used in systematic studies to resolve divergence at many taxonomic levels. Previous research used this locus for genetic study of Eurasian badgers (*Meles* sp.). The mitochondria indicated a great divergence between the badgers from Eastern and Central Europe, Siberia, and Japan (Kurose *et al*., 2001; Sato *et al*., 2003). This research showed that the superfamily Musteloidea emerged approximately 32.4–30.9 MYA in Asia, shortly after the greenhouse-icehouse global climate shift during the Eocene-Oligocene transition (Sato *et al*., 2012).

In conclusion, teledu can be confirmed as one of the species in the family Mephitidae, and specifically, the genus *Mydaus*. This study also supports the molecular data, which showed that *Mydaus* forms a lineage with the family Mephitidae. In addition, both 12S rRNA and Cyt *b* are effective in teledu's systematics, as these loci can estimate rates of species divergence and determine the relationships in families and genera (Abdul-Latiff *et al*., 2014b; Abdul-Latiff *et al*., 2017; Rosli *et al*., 2011a,b).

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