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Molecular Phylogenetics and Phylogeography of Malaysian Mousedeer (*Tragulus kanchil*) Based on Mitochondrial DNA Sequences of the D-Loop Region

(Filogenetik Molekul dan Filogeografi Pelanduk Malaysia (*Tragulus kanchil*) Berdasarkan Jujukan DNA Mitokondria Kawasan D-Loop)

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ABSTRACT

Two sympatric mousedeer species, *Tragulus kanchil* and *Tragulus napu,* inhabit the Malaysian tropical rainforests. Previous studies have established their phylogenetic relationships based on morphological variations; however, comprehensive genetic data have yet to be extensively used to relate the relationships especially from different populations. The main objectives of this study were to determine the phylogenetic relationships, population genetics, and phylogeography of mousedeer species based on DNA sequences of the mitochondrial D-loop region. DNA from 32 mousedeer samples, representing various populations in Malaysia, was sequenced and analyzed using Neighbor-Joining, Maximum Parsimony, and Bayesian Inference approaches. The phylogenetic analyses showed two main clades representing the populations of *T. kanchil* and *T. napu*. The results also showed that the *T. kanchil* populations in Borneo was separated from Peninsular Malaysia taxa in MP and BI phylogenetic tree. However, Borneo population was nested in east population of Peninsular Malaysia in NJ tree. In addition, the *T*. *kanchil* population in Peninsular Malaysia further separated into east and west coast populations of Titiwangsa Range. It was supported with finding in population genetic relation that showed relatively low levels among population. This is expected because some of the populations are isolated geographically. The divergence of these populations is likely due to the Titiwangsa Range which acts as a barrier separating the east and west Peninsular Malaysia populations, and the South China Sea separates the populations of Peninsular Malaysia and Borneo. Molecular clock tree reconstruction showed that the separation of *T. kanchil* and *T. napu* occurred around 17.11 million years ago (MYAs). Furthermore, the *T. kanchil* populations from the east and west Peninsular Malaysia showed a branching pattern from those of Borneo of about 11.04 and 9.14 MYAs, respectively. The results of this study increase our understanding of Malaysian mousedeer phylogeny and phylogeography.

Keywords: Mousedeer; phylogeography; *Tragulus kanchil*; *Tragulus napu*; ungulate

ABSTRAK

Dua spesies pelanduk *Tragulus kanchil* dan *Tragulus napu,* bersimpatrik menghuni hutan hujan tropika Malaysia. Kajian terdahulu telah mengesahkan pertalian filogenetik pelanduk berdasarkan variasi morfologi. Walau bagaimanapun, data genetik yang komprehensif masih belum digunakan sepenuhnya untuk mengaitkan hubungan terutamanya populasi yang berlainan. Objektif utama kajian ini adalah untuk menentukan pertalian filogenetik, genetik populasi dan filogeografi kedua-dua spesies pelanduk menggunakan jujukan DNA mitokondria kawasan D-loop. DNA daripada 32 sampel pelanduk yang mewakili populasi di Malaysia telah dijujuk dan dianalisis menggunakan kaedah *Neighbor-Joining (NJ), Maximum Parsimony (MP)* dan *Bayesian Inference* (BI). Analisis filogenetik menunjukkan terhasil dua klad utama mewakili populasi *T. kanchil* dan *T. napu*. Keputusan juga menunjukkan populasi *T. kanchil* di Borneo terpisah daripada populasi Semenanjung Malaysia berdasarkan pokok filogenetik MP dan BI. Walau bagaimanapun, populasi Borneo masih tergolong dalam satu kumpulan bersama populasi Timur Semenanjung Malaysia dalam pokok NJ. Selain itu, populasi *T. kanchil* di Semenanjung Malaysia terpisah kepada populasi barat dan timur Banjaran Titiwangsa. Ini juga disokong dengan hasil analisis populasi genetik menunjukkan tahap hubungan yang rendah antara populasi. Hal ini dijangka disebabkan populasi ini terpisah oleh halangan geografi. Pencapahan populasi ini berkemungkinan disebabkan Banjaran Titiwangsa bertindak sebagai penghalang yang memisahkan populasi Timur dan Barat Semenanjung Malaysia dan Laut China Selatan yang memisahkannya dengan populasi Borneo. Pohon jam molekul juga menunjukkan pemisahan spesies *T. kancil* dan *T. napu* berlaku sekitar 17.11 juta tahun dahulu (JTD). Tambahan lagi, populasi *T. kancil* Timur dan Barat Semenanjung Malaysia menunjukkan percambahan daripada populasi Borneo masing-masing sekitar 11.04 JTD dan 9.14 JTD. Hasil kajian ini dapat meningkatkan pemahaman tentang filogeni dan filogeografi spesies kancil di Malaysia.

Kata kunci: Filogeografi; pelanduk; *Tragulus kanchil*; *Tragulus napu*; ungulat

Introduction

The superorder Cetartiodactyla includes 22 families, 130 genera, and 330 species (Vislobokova 2013). One of the taxonomic groups within Cetartiodactyla is Artiodactyla, which consists of the following three suborders: Ruminantia, Suiformes, and Tylopoda. In Ruminantia, we find the *Tragulus* spp., commonly known as mousedeer, a forest ungulate native to Asia and Africa (Matsubayashi & Sukor 2005; Meijaard & Groves 2004). In Southeast Asia (SEA), mousedeer is divided into the following three major species: *Tragulus javanicus*, *T. napu*, and *T. versicolor* (Meijaard & Groves 2004). Recently, *T. javanicus* was further revised into two species, *T. kanchil* and *T. javanicus* (Timmins & Duckworth 2015), a separation first suggested by Meijaard and Groves (2004), who investigated the Javan population and identified distinct morphological features of the two mousedeer populations.

Asian mousedeer is distributed broadly in SEA, including Malaysia, Thailand, Myanmar, Cambodia, Laos, Vietnam, and Indonesia (Corbet & Hill 1992). In Malaysia, there are two mousedeer species that can be identified by their body size, *T. kanchil* (Lesser mousedeer) and *T. napu* (Greater mousedeer). Although these two species generally differ in average size, misidentification sometimes occurs because they are geographically sympatric and have an overlapping variation in body size. Compounding the confusion, morphological characteristics such as coat color, body size, and skeletal structure do not provide clear distinctions between these two species (Corbet & Hill 1992; van Dort 1988).

Tragulus kanchil and *T. napu* are categorized as least-concern species on the International Union for Conservation of Nature Red List of Threatened Species (Timmins & Duckworth 2015) and are listed as protected species under the Wildlife Conservation Act 2010 [Act 716]. Regardless of their status, mousedeer are positive indicators of environmental health because they preserve balance in their habitat communities. As prey, they sustain carnivores, and as herbivores, they disperse seeds and enhance soil fertility (Khalil et al. 2019).

Molecular studies could contribute to information on the phylogenetic relationships and evolutionary history of *Tragulus* species. Currently, molecular studies are lacking and this can hinder conservation interventions and cause the loss of unique genetic lineages in Malaysia. Molecular systematic studies have been useful in

investigating the phylogenetic relationships of other mammalian groups and in differentiating subspecies that are difficult to identify using only external characteristics (Aifat, Yaakop & Md-Zain 2016; Kim et al. 2004; Rosli et al. 2011).

Very few studies focus on the genetic relationships of *Tragulus* species. Endo et al. (2004) employed cytochrome *b* gene and discovered three clusters of mousedeer in mainland Asia (Malaysia and Laos) as well as Borneo, while Wirdateti and Nugraha (2016) employed 12S rRNA to evaluate the genetic variation in Indonesian mousedeer. Other studies focused on the physiology, histology, and biology of *Tragulus* (Farida et al. 2006; Kusuda et al. 2013; Matsubayashi & Sukor 2005; Meijaard, Umilaela & de Wijeyeratne 2010). Due to lack of molecular studies in *Tragulus* species, especially in Malaysia, their phylogenetics, population genetics, and phylogeography remain unknown. Thus, the main objectives of this study were to determine the Malaysian mousedeer (*Tragulus kancil*) phylogenetic relationships and their radiation history using the D-loop region of mitochondrial DNA (mtDNA). In addition, we also added samples from its close species, *Tragulus napu,* for comparison purposes. The D-loop region was selected as a candidate locus as it has been successfully employed in other mammalian molecular systematics and population genetic studies (Abdul-Latiff & Md-Zain 2021; Abdul-Latiff et al. 2014a, 2014b; Aifat et al. 2020). D-loop is a non-coding region section of the mitochondrial genome that stores the mitochondrial origins of transcription and replication. D-loop is a preferred marker sequence for genetic diversity analyses due to several distinct characteristics. These characteristics include its polymorphic nature, and selective neutrality beside its general advantages of mitochondrial such as maternal inheritance, lack of recombination, and fast rate of evolution. In addition, the D-loop is a preferred marker due to its exceptionally fast evolutionary rate compared to other genes of the mitochondrial (Abdul-Latiff et al. 2019; Abdul-Patah et al. 2020).

Materials and Methods

DNA extraction, polymerase chain reaction (PCR), and DNA sequencing

Thirty genetic samples were collected from across mainland Peninsular Malaysia and Borneo (Table 1; Figure 1). Muscle tissue and blood samples of *Tragulus kanchil* and *Tragulus napu* were procured from dam development zones in Terengganu (Tembat and Puah Dam), prior to the relocation of animals to a safer habitat in the adjacent forest. Some of the samples were provided by the Department of Wildlife and National Parks in Malaysia (Jabatan PERHILITAN) and Jabatan Hutan Sarawak. Seven sequences of *T. javanicus* and *T. napu* data were retrieved from GenBank for comparative analysis The Invisorb Spin Tissue Mini Kit (STRATEC Molecular, Berlin, Germany) was used to extract DNA from muscle tissue, while the DNeasy Blood and Tissue Kit (QIAGEN, Valencia) was used for blood samples (FTA card), following the manufacturers' protocols (Syed-Shabthar et al. 2013).

PCR was performed using MyTaq™ Red Master Mix (Bioline, Country) in a Mastercycler® Nexus (Eppendorf North America, Inc.). Universal primers L15926 (5'-TCAAAGCTTACACCAGTCTTGTAAACC-3'), L16007 (5'-CCCAAAGCTAAAATTCTAA-3'), and H00651 (5'-TAACTGCAGAAGGCTAGGACCAAACCT-3') were used to target the mtDNA D-loop region for all samples (Kocher et al. 1989). The PCR reaction conditions were as follows: 98 °C for initial denaturation for 10 s, followed by 30 cycles of 98 °C for denaturation for 1 s, 51 °C for annealing for 30 s, 72 °C for extension for 15 s, and a final extension stage at 72 °C for 1 min. The PCR amplification products of both strands (forward and reverse) were sent to Apical Scientific Sdn. Bhd. (Malaysia) for DNA sequencing.

Phylogenetic, population, and phylogeography analyses

The DNA sequences from the samples were edited using BioEdit Sequence Alignment Editor and aligned using ClustalW together with the DNA sequences retrieved from GenBank (Aifat & Md-Zain 2021). The edited sequences were confirmed using similarity searches (GenBank BLASTn) to ensure that the targeted species and loci were correctly obtained. Five DNA sequences for each of *T. javanicus* (3 sequences) and *T. napu* (2 sequences) were retrieved from GenBank and used for comparative phylogenetic analysis. Several phylogenetic tree reconstruction methods were performed, including a distance-based method (Neighbor-joining, NJ), character-based method (Maximum Parsimony, MP) using MEGA X (Chowdhury et al. 2003; Halim et al. 2018; Kumar et al. 2018). Bayesian inference (BI) using MrBayes 3.1 (Huelsenbeck & Ronquist 2001), and *Hyemoschus aquaticus* (Accession number: NC 020714.1) was used

TABLE 1. List of *Tragulus* genetic samples

Species	Locality	Samples (Accession number)
1. *Tragulus kanchil*	Terengganu (Puah and Tembat)	TJET3 (OP594879) TJET8 (OP594880) TJET10 (OP594881) TJET4 (OP594882) TJET5 (OP594883) TJET14 (OP594884) TJET15 (OP594885) TJEP16 (OP594886) TJEP5 (OP594887) TJEP7 (OP594888) TJEP2 (OP594889) TJEP17 (OP594890) TJEP3 (OP594891) TJEP18 (OP594892) TJEP8 (OP594893)
	Johor (Kahang)	TJ21 (OP594894) TJ85 (OP594897)
	Selangor (Dengkil)	TJ16 (OP594878) TJG21 (AF266572.1) (GenBank) TJG20 (AF266573.1) (GenBank)
	Perlis (Pusat Konservasi Hidupan Liar Perlis, Sungai Batu Pahat)	TJ90 (OP594874) TJ34 (OP594875) TJ91 (OP594876) TJ36 (OP594877)
	Pahang	TJ30 (OP594895) TJ29 (OP594896)
	Sarawak (Pueh, Tanah Rata)	TJQ32 (OP594870) TJQ29 (OP594871) TJQ2 (OP594872) TJQ30 (OP594873) TJG5 (AF266688.1) (GenBank)
2. *Tragulus napu*	Perlis (Pusat Konservasi Hidupan Liar Perlis, Sungai Batu Pahat)	TN59 (OP594899)
	Kedah (Langkawi)	TN49 (OP594898)
	Johor	TNG8 (AF263994.1) (GenBank) TNG6 (AF263996.1) (GenBank)
	Pahang	TNG2 (AF264000.1) (GenBank) TNG1 (AF264001.1) (GenBank)

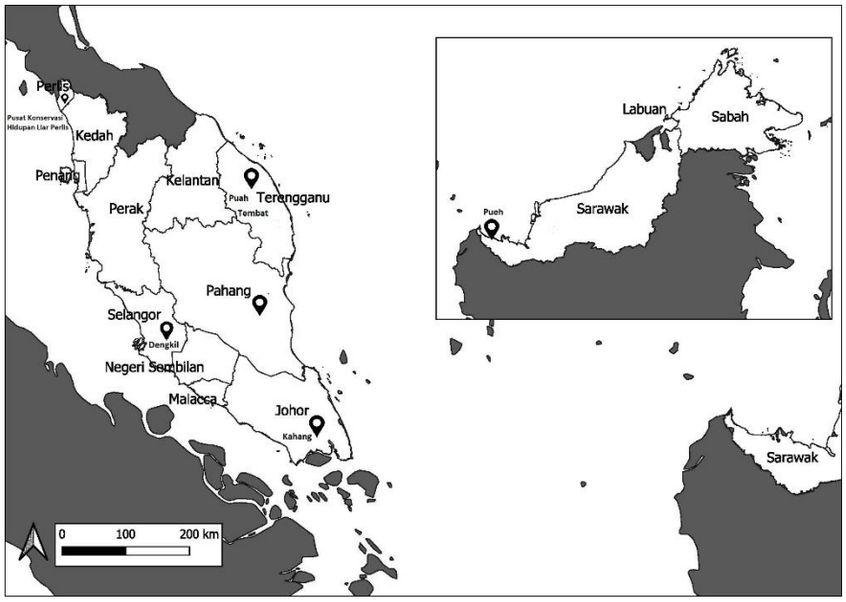

FIGURE 1. Origin of the genetic samples

as an outgroup as the most recent common ancestor. NJ tree reconstruction was accompanied by bootstrap analyses with 1000 replications employing the Kimura-2-parameter model. For MP analyses, the heuristic searching method and 1000 random stepwise additions were applied to find the best tree (Md-Zain et al. 2010). For Bayesian analysis, the best substitution model was determined using Akaike Information Criterion Modeltest version 3.7 software (Posada & Crandall 1998). The most suitable model that fits the data was the HKY + I + G model with a gamma shape parameter of 0.6300 and base frequencies of 0.3048 for A, 0.2597 for C, 0.1138 for G, and 0.3018 for T. We ran Metropolis-coupled Markov chain Monte Carlo (MCMC) with 1,000,000 generations, 0.006355 split frequencies Probability (P), with a tree sampled every 100 generations. The first 25% of the trees obtained in the analysis were discarded as burn-ins (2,500 trees discarded from a total 1,000,000 trees). A majority-rule consensus of the remaining trees was reconstructed, and posterior probabilities (PP) were calculated for each branch.

A molecular clock tree was reconstructed using an uncorrelated lognormal relaxed-clock model. This analysis was performed using BEAST v1.7.5 software to estimate the divergence dates of the *T. kanchil* and *T. napu* populations in Malaysia (Drummond & Rambaut 2007). *Hyemoschus aquaticus* (Accession number: NC 020714.1) was used as a calibration point as the most recent common ancestor, estimated around 22.5 million years ago (MYAs), based on genetic dataset calibrations from the previous study (Hassanin et al. 2012). Other population genetic parameters were also estimated: nucleotide diversity (π), nucleotide divergence (Da), nucleotide subdivision (Nst), population subdivision (F_{st}), number of migrants per generation (N_m) and haplotype analyses using Arlequin 3.5 and DnaSP 4.0 (Mohd-Yusof et al. 2020; Rozas et al. 2017). Network 5.0 was used to generate a minimum-spanning network (MSN) to portray the relationships of each distinguished haplotype in the *Tragulus* species.

Results

DNA sequences of 889 bp were obtained from the D-loop region. Within this fragment, 664 characters were conserved and 225 were variable sites. In addition, 97 were autapomorphic and 128 were parsimony informative. The average pairwise distance based on the Kimura-2-parameter model for the population of *T. kanchil* in Terengganu, Selangor, Johor, Pahang, Perlis, and Sarawak was 0.006–0.026 (Table 2). Within Peninsular Malaysia, a far genetic distance was observed between the Terengganu and western and northern populations (Selangor and Perlis), as high as 0.024 and 0.019, respectively. The Selangor population also

TABLE 2. Average pairwise distances among *Tragulus* populations based on the Kimura-2 parameter

Population	[1]	[2]	[3]	[4]	[5]	[6]
[1] Terengganu						
[2] Selangor	0.024					
[3] Johor	0.010	0.021				
[4] Pahang	0.010	0.017	0.006			
[5] Perlis	0.019	0.008	0.016	0.012		
[6] Sarawak	0.022	0.026	0.020	0.017	0.023	
[7] *T. napu*	0.125	0.120	0.122	0.118	0.120	0.118

showed a high genetic distance from the southern population (Johor), but a low distance was observed when compared with the northern population (Perlis), with 0.021 and 0.008, respectively. The pairwise distance between *T. kanchil* and *T. napu* showed a high genetic distance of 0.118–0.125.

The NJ, MP, and BI (Figures 2-4) phylogenetic trees showed parallel results. The *T. kanchil* clade is clearly distinguished from *T. napu* with a high bootstrap value of 99%, 100%, and a 1.0 PP, for NJ, MP, and BI, respectively. *Tragulus kanchil* populations from Peninsular Malaysia were separated into 2 clades; clade A and clade B (NJ tree). Clade A portrays the separation of samples originating from the east of Titiwangsa Range, Peninsular Malaysia from the remaining samples with 61% (NJ tree) bootstrap support. On the other hand, Clade B is assemblage of populations that reside in the west of Titiwangsa Range, Peninsular Malaysia (Selangor and Perlis) with bootstrap supported by 68% (NJ). However, there were two Terengganu samples from east group found in west group of Titiwangsa Range based on MP and BI trees (Figures 3 & 4). In addition, the *T. kanchil* clade can be seen clearly separated into the Peninsular Malaysia and Borneo regions only for MP and BI phylogenetic trees. The separation of Borneo clade from Peninsular Malaysia taxa was supported by 74% (MP) bootstrap support and 0.9 PP (BI). However, in NJ tree, the Borneo population was grouped in Peninsular Malaysia with moderate bootstrap value support of 60% (Figure 2). For the molecular clock tree, it indicated that the *T. kanchil* and *T. napu* populations diverged from *H. aquaticus* approximately ~21.63 MYAs and that *T. kanchil* and *T. napu* separated around ~17.11 MYAs. For the *T. kanchil* species, the Peninsular Malaysia population diverged from the Borneo population ~11.04 MYAs, followed by a separation of the eastern and western coast populations ~9.14 MYAs (Figure 5).

Twenty-one *T. kanchil* haplotypes were recognized from haplotype analyses. The population in Terengganu was determined to have 11 haplotypes, Hap_1–Hap_11. The population in Sarawak has four haplotypes, Hap_18–Hap_21. Meanwhile, the populations in Selangor and Johor were each determined to have two haplotypes, Hap_12–Hap_13 and Hap_ 14–Hap_15, respectively. The Pahang and Perlis populations had only one haplotype each, Hap16 and Hap17, respectively. An MSN portrayed the relationships among the haplotypes (Figure 6). The populations in Terengganu were separated by three mutational sites, and those from Johor and Pahang were separated by four mutational sites. Although Johor is located in the southern part of Peninsular Malaysia, phylogenetic analysis indicates that it is genetically grouped with the eastern region of the Peninsular Malaysia. The populations in Terengganu show a relatively small genetic difference, with only three mutation sites, while the populations in Pahang exhibit slightly higher divergence, with four mutation sites toward Johor population in haplotype analysis. This is consistent with the low population structure observed, as measured by F_{ST} values. Analysis of the D-loop region sequences also showed a low average number of genetic changes per site, suggesting that the observed mutations

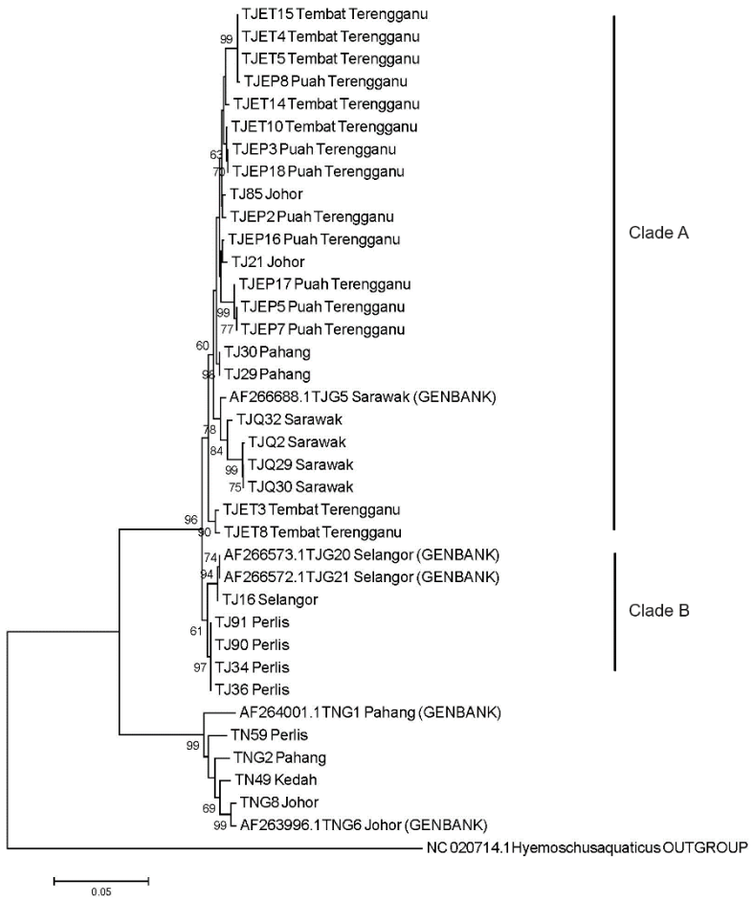

FIGURE 2. Neighbor Joining tree estimated using the Kimura-2-parameter algorithm and 1,000 bootstraps replications (value <50% is not shown)

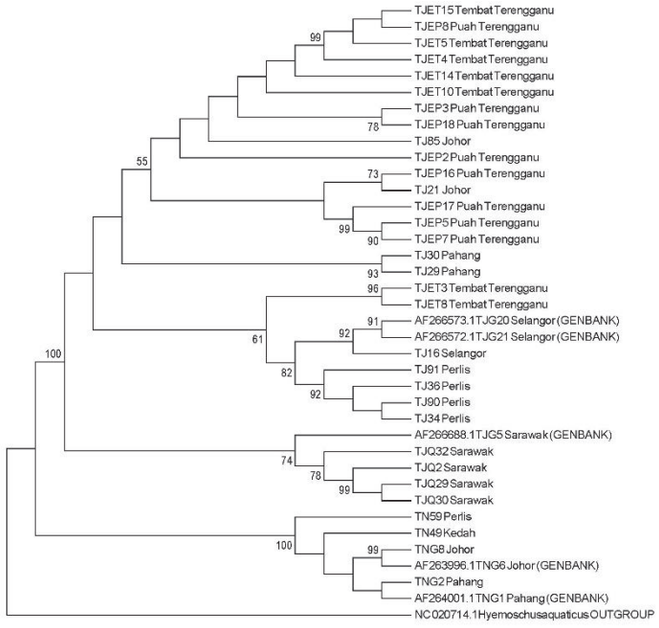

FIGURE 3. Maximum Parsimony phylogenetic tree estimated using the TBR algorithm, heuristic searching method, and 1,000 bootstrap replications (value <50% is not shown)

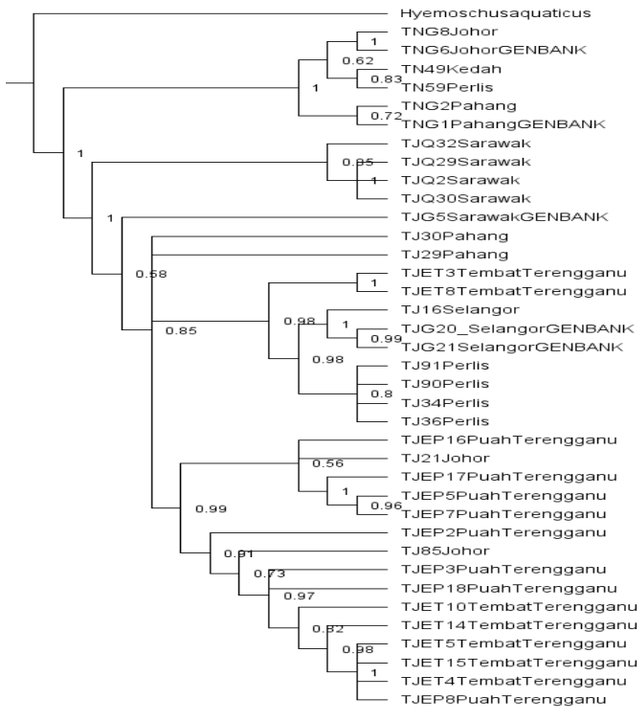

FIGURE 4. Bayesian inference (BI) tree of the 50% majority-rule consensus and the molecular divergence tree of the D-loop sequence of *Tragulus* spp. with Bayesian posterior probability (PP) indicated on the branches

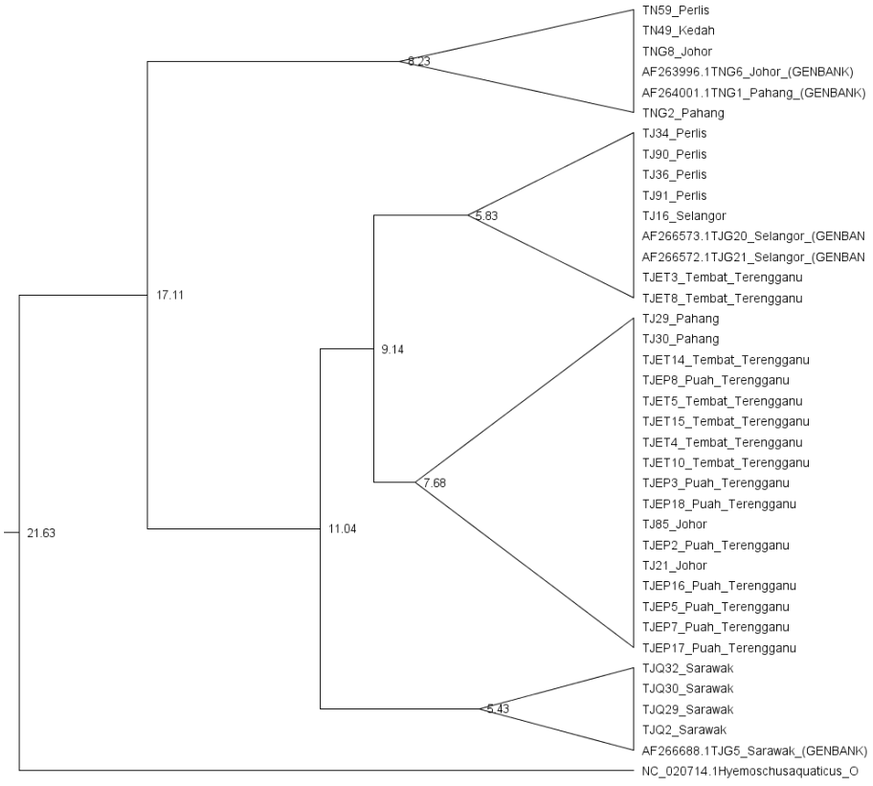

FIGURE 5. The molecular clock tree of *Tragulus* spp. populations with numbers on the nodes representing divergence time in MYAs

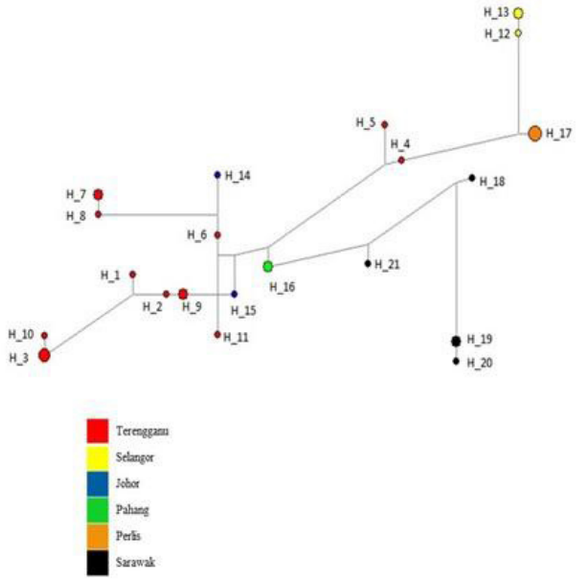

FIGURE 6. MSN of the *T. kanchil* in Malaysia

in Johor, Terengganu, and Pahang populations are more likely due to historical factors rather than geographical isolation. Furthermore, our study shows a high level of gene flow between the Johor and Terengganu populations, indicated by a substantial migration rate (Nm = 0.89). This suggests ongoing genetic exchange and contributes to the overall weak population differentiation within the states. These findings highlight the dynamic nature of genetic connectivity and indicate a continuous exchange of genetic material among these populations. Meanwhile, the population in Sarawak was separated from the east coast population by seven mutational sites. The Selangor and Perlis populations had a close relationship with each other and were separated from other populations by seven mutational sites.

Nucleotide diversity (π) and net nucleotide divergence (Da) were obtained using DnaSP 4.0. The highest π value was recorded between the Borneo population and the west population of Peninsular Malaysia, ranging from 0.01520 to 0.01690 (Table 3). The result was consistent with Da, ranging from 0.01870 to 0.02090. The π and Da values were much lower between the Borneo population and the east population, ranging from 0.0120 to 0.01557 and 0.01140 to 0.01231, respectively.

Genetic differentiation (F_{ST}, N_{st}, and N_m) values were calculated to further elucidate the relationships among T. kanchil populations from six different Malaysian states (Table 3). *Tragulus kanchil* populations from all six states showed a subdivision from the Borneo population with a minimum value of F_{ST} of 0.53813 (Terengganu–Sarawak) and a maximum value of 0.81602 (Selangor–Sarawak). Between states in the Peninsula, the lowest F_{ST} value was 0.01095 between the Terengganu and Johor populations. In contrast, the Pahang and Perlis populations had the highest division with 1.00000. Nst analysis was consistent with F_{ST} value, with the Terengganu and Johor populations having the lowest Nst value of 0.01050, followed by the Pahang and Johor populations with 0.36293. The Pahang and Perlis populations had the highest Nst value at 1.0000. The N_m value should be inversely proportional to both F_{ST} and Nst. N_m analysis validated both F_{ST} and Nst, as the Borneo population had quite a low N_m value from Peninsular Malaysia, ranging between 0.11 and 0.42. Within Peninsular Malaysia, the lowest N_m value was between Pahang and Perlis (0.00), and the highest N_m value was between Terengganu and Johor (0.89).

TABLE 3. Measures of π, Da, estimate of F_{ST}, Nst, and N_m among populations of *T. kanchil*

Population	π	Da	F_{ST}	Nst	N_m
Terengganu-Pahang	0.01125	0.00444	0.43280	0.43123	0.66
Terengganu-Selangor	0.01488	0.01719	0.73487	0.73665	0.18
Terengganu-Perlis	0.01374	0.01296	0.68999	0.69099	0.22
Terengganu-Johor	0.01123	0.00011	0.01095	0.01050	0.89
Terengganu-Sarawak	0.01557	0.01183	0.53813	0.54106	0.42
Pahang-Selangor	0.01026	0.01634	0.97727	0.97751	0.01
Pahang-Perlis	0.00608	0.01140	1.00000	1.00000	0.00
Pahang-Johor	0.00551	0.00228	0.36364	0.36293	0.88
Pahang-Sarawak	0.01205	0.01231	0.73973	0.74076	0.17
Selangor-Perlis	0.00445	0.00722	0.95000	0.95022	0.03
Selangor-Johor	0.01345	0.01634	0.78899	0.79089	0.13
Selangor-Sarawak	0.01690	0.02090	0.81602	0.81786	0.11
Perlis-Johor	0.00874	0.01140	0.74074	0.74204	0.17
Perlis-Sarawak	0.01520	0.01870	0.81188	0.81333	0.11
Johor-Sarawak	0.01390	0.01140	0.57803	0.58108	0.36

DISCUSSION

The NJ, MP, and BI tree topologies clearly separated *T. kanchil* and *T. napu* into their own monophyletic groups. The average pairwise distance also showed a clear distant relationship between these two species. Thus, we were able to distinguish *T. kanchil* and *T. napu* at the molecular level, the difficulties in the previous studies in reconstructing their classification (Kuznetsov & Borissenko 2004; Meijaard & Groves 2004). With the advent of molecular genetic techniques, the classification and taxonomy resolution in mousedeer has improved. Our molecular results confirm the traditional taxonomic separation of *T. kanchil* and *T. napu* (Meijaard & Groves 2014) and support previous taxonomy assignments based on morphological characteristics to distinguish between these two taxa (Corbet & Hill 1992; Meijaard & Groves 2004). Our findings also support Wirdateti and Nughara (2014), who were able to separate these two species using 12S rRNA in Indonesian populations. Thus, our selected candidate locus, the mtDNA D-loop region, was an effective locus for resolving the phylogeny of *T. kanchil* and *T. napu* in Malaysia.

The presence of anomalies in our results, particularly the mixed samples within different clades, can be attributed to the effects of wildlife trade and the subsequent removal and reintroduction of individuals from their original populations (Abdul-Latiff & Md-Zain 2021). Wildlife trade involving *T. kanchil* is a pervasive issue, driven by the demand for its meat and parts (Nur-Syuhada et al. 2016). As a result, individuals are often captured and removed from their natural habitats, leading to disruptions in gene flow and population structure. The subsequent reintroduction of these individuals into new populations, whether intentional or accidental, can

contribute to the mixing of genetic lineages and the observed anomalies in our data. Despite the uncertainties arising from the mixed samples, it is crucial to note that our study's primary focus is on establishing the hypothesis of east-west and Borneo-Peninsular separation. We aim to identify and define evolutionary significant units (ESUs) within the species, as this information holds significant implications for conservation genetics. By understanding the population structure and potential genetic differentiation between regions, we can develop targeted conservation strategies that account for the unique gene pools and evolutionary trajectories within each population. While the presence of mixed samples may complicate the analysis, it reinforces the urgency and necessity of investigating the population dynamics and genetic connectivity to effectively conserve the species and its evolutionary heritage.

Tree topologies (MP and BI) and average pairwise distant values indicated that *T. kanchil* in Malaysia is divided into two clades, where the Borneo population was separated from Peninsular Malaysia populations. However, NJ tree was not portrayed as the same topologies in which the Borneo population was nested within Peninsular Malaysia population. Furthermore, the Peninsular Malaysia populations were further divided into two subclades, the east and west groups of Titiwangsa Range. We expected the populations in Peninsular Malaysia and Borneo to separate with higher values throughout the analyses. However, the NJ clade was not statistically supported. Even though the D-loop region is acknowledged as the mitochondria's fastest evolving area, sometimes it gives unclear true phylogenetic relationships especially toward species that exhibit male-biased dispersal and interspecific genes (Kutchera et al. 2014). Analyses based on a single gene may be restricted and inaccurate due to poor lineage sorting. The usage of various nuclear and mitochondrial markers may provide a full picture of species phylogeny (Rubinoff & Holland 2005). Despite these limitations, this work contributes significantly to understanding the evolutionary history of *Tragulus* species in Malaysia and indicate possible gaps in our understanding as well as the importance of using multiple genes or complete genome.

Nevertheless, the results showed some significant findings in relation to population genetic variation of *T. kanchil*; it was observed that there are relatively low levels of between-population variation among populations, as evidenced by the π and Da values (Table 3). This observation was expected because some of the individuals are isolated geographically. Titiwangsa Range, the main mountain range in Peninsular Malaysia, effectively isolates the eastern and western states, suggesting that natural gene flow between the states is reduced. As such, if gene flow occurs, then this is likely due to human-mediated translocations as seen in the NJ tree result where some samples from the east group were found in the west group. The genetic diversity and genetic differentiation results from this study indicated that the Peninsular Malaysia populations of *T. kanchil* are strongly distinct from the Bornean population, as evidenced by the high genetic distance, low nucleotide diversity, and net nucleotide diversity. The nucleotide subdivision indices also displayed a parallel pattern, with the highest subdivision and lowest gene flow between these two regions. Likewise, the low level of gene flow between the *T. kanchil* populations of these two regions may be associated with the isolation of Borneo. Geographical isolation is suspected to be the reason for the genetic separation of the Borneo population from the Peninsular Malaysia populations. Geographical isolation and constraints in migratory can disrupt gene flow between two populations and affect the long-term persistence of population (Brunke et al. 2020). The isolation of a small population from the main population may also produce genetic drift.

F_{ST} shows population differentiation due to genetic structure. If the value of F_{ST} is greater than 0.15, then it is considered significant in differentiating a population (Frankham, Ballou & Briscoe 2002). Thus, a significant divergence was found within each *T. kanchil* population according to the F_{ST} value obtained from Arlequin 3.5 (Table 3). High F_{ST} values indicate significant genetic divergence, probably due to the low exchange of genetic material between populations. An N_m value close to 1 indicates a limited gene exchange between subpopulations (Wright 1965). In this study, the N_m values ranged from 0.01-0.89, suggesting that gene flow may occur and lead to a low genetic differentiation between subpopulations for some states. Populations from the same region, i.e., east and west regions of Peninsular Malaysia, were the least variable genetically (Table 3), which is likely due to the close geographical distance between these populations. Within the Peninsular Malaysia population, a moderate level of genetic variation was observed. A high gene flow was observed within the west and east coast populations. In contrast, the west and east regions had high genetic differentiation values and the lowest gene flow. This indicates strong population structuring with large geographical barriers and distance. The significance

value of genetic variation within a population may be influenced by dispersal events and is expected to increase with increased distance between populations (Mohd-Ridwan & Abdullah 2012). A high level of haplotype diversity was recorded from sequence analysis of the *T. kanchil* populations in the Peninsular Malaysia and Borneo regions. All haplotypes were considered unique because none were shared across localities. This single-locality, unshared haplotype suggests that there is a large geographical barrier preventing gene flow (Whitlock & McCauley 1999). Our study also agreed with Meijaard and Groves (2004), who showed that there is a difference in cranial morphometric data between the Peninsular Malaysia, and Borneo populations.

Investigating variation in mitochondrial genomes across the *T. kanchil* distribution range enabled us to examine the evolutionary history of these populations, including their phylogeography structure. The radiation of *T. kanchil* possibly began from the Indochinese region and extended to another region in the Sunda shelf. This hypothesis is based on the oldest *Tragulus* fossil encountered in Krabi, Thailand, the *Archetragulus krabiensis*, which is estimated to be from the late Eocene period (Mètais et al. 2001). Mètais et al. (2001) believed that Thailand represents the oldest occurrence of the family *Tragulus* and that early diversification of ruminants occurred in SEA. According to the fossil record, tragulid species underwent a wide radiation during the early Miocene throughout Africa, Europe, and South Asia (Mètais et al. 2007). Based on our phylogenetic reconstruction and estimated divergence time, the *T. kanchil* population initially split into two clades (Peninsular Malaysia and Borneo populations), 11.04 MYAs in the late Miocene period. During this period, SEA underwent a major geographical change (Hall 2013, 2002). At that time, there were lower sea levels than presently, about 50-60 m below current day levels, and fewer areas of contemporary geography were connected (Houben et al. 2012; Voris 2000). In the late Miocene, some parts of the Sunda shelf were linked by land bridges and formed a single mainland (Hall 2001). It is probable that tragulids distributed throughout this landmass became gradually isolated as the sea level rose (Miller et al. 2005). Furthermore, Earth underwent climatic changes that transformed the forest environment in SEA. These environmental changes caused some mammals to expand their range of dispersal to areas that suited their preferences (Harrison & Chivers 2007) and could be a factor in the migration and expansion of the tragulid species during the early to late Miocene. Nevertheless, the status of the regional phylogenetic separation needs to be validated. In Peninsular Malaysia, the dispersal continues, and the population appears to be undergoing another separation into the southeast and northwest populations. We hypothesize that the Banjaran Titiwangsa mountain range is causing the tragulid population to diversify into two separate populations. Previous studies in several species have shown that Titiwangsa mountain is a natural barrier that can cause differentiation (Hurzaid et al. 2014; Kamaruddin & Esa 2009; Loo et al. 2001; Sum et al. 2014). Overall, any attempt to reconstruct biogeographic history in this region will depend on the existence of fossil evidence. Future studies on the geographic distribution pattern within species are recommended to help increase the understanding of the geological and climatological history of the region.

Conclusion

The phylogenetic and phylogeography of Malaysian mousedeer populations were studied. The molecular data supported the morphological characteristics that indicated *T. kanchil* and *T. napu* were different. Among the *T. kanchil* populations, tree topologies showed separation of two distinct groups east and west populations of Titiwangsa Range. Even the tree topologies showed unclear separation between Peninsular Malaysia's and Borneo's populations but population genetic analyses, indeed helped to distinguish genetic variation and population differentiation among populations. This study shows the usefulness of the mtDNA D-loop region in assessing mousedeer phylogenetic relationships. More genetic markers, such as nuclear genes or even complete mtDNA genomes, should be used to verify the Malaysian mousedeer taxonomic status and radiation pattern.

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