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# Phylogenetic relationship of mouse deer from the Southern of Vietnamassessed by Cytochrome b

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***Abstract***

***This study aimed to assess the phylogenetic relationship of Vietnamese mouse deer from the Southern ofVietnam using cytochrome b. Eleven mouse deer samples (CT1 to CT9, and LD1-LD2)from Lam Dong Province and Cat Tien National Park of DongNaiProvince were used for this investigation. The sequence analysis revealed 37 polymorphic sites,in which 13 variable positions (14328, 14337, 14400, 14427, 14436, 14466, 14523, 14526, 14530, 14537, 14541, 14544, 14550) were observed in Vietnamese mouse deer.Moreover,Vietnamese mouse deer showed the lowest genetic distance withTragulusjavanicus (0.031 ± 0.014)compared toTragulusnapu (0.051 ± 0.022)andTraguluskanchil (0.035 ± 0.016). Phylogenetic tree building showed that Vietnamese mouse deer were clustered in distinct group with 100% bootstrap probability. In addition, the variable position at 14325 (A → G) contributed to the separation of CT2, CT4, CT7, and CT8 in one group with 64% bootstrap probability.***

***Keywords:cytochrome b, mouse deer, phylogeny.***

## Introduction

To analyze the phylogenetic relationships among various animal groups, molecular techniques have been employed. The most popular and well known method of understanding the candidate's evolutionary genetic information is through DNA sequence analysis. Among all, mitochondrial DNA (mtDNA) has been widely used to perform phylogenetic studies in different animal species for several reasons. The advantage of mitochondrial DNA over other organelles is due to that evolution of mammalian mtDNA occurs primarily as single base pair substitutions, with only infrequent major sequence rearrangements (Wolstenholme, 1992). Also, the rate of mtDNA

evolution appears to be as much as 10 times faster than that of nuclear DNA (Brown et al., 1979). Moreover, mtDNA is maternally inherited, haploid and non-recombining (Avise et al., 1994). As a result, mtDNA is thus served as the genetic marker to investigate the evolution studies based on some several favorable characteristics including large quantity in the cell, small genome size, maternal inheritance and extremely low probability of paternal leakage (Cummin et al.,1997), higher mutation rate than nuclear DNA, and change mainly through mutation rather than recombination (Eyre-Walker and Awadalla, 2001).

The mouse-deer (Tragulidae) are an ancient group of ungulates (Meijaard and Groves, 2004). In southern Asia, they are found in fossil assemblages dated at 18 million years before present (YBP), although they reached their highest diversity with five named and 52 unnamed species at around 11.5 YBP (Barry et al., 1991). mtDNA analysis has been recently used for evaluation of genetic relationship of mouse deer. The mtDNA analysis of *Moschiolaindica* clustered the *Tragulidae* as sister-group of all other ruminants. *Moschiola* forms the sister-group to the other two tragulid genera *Tragulus* (from Asia) and *Hyemoschus* (from Africa) (Sarvani et al., 2018).

The genetic structure among populations of *Tragulusjavanicus* has been reported by Meijaard and Groves (2004), who revealed the morphological differences of mouse deer from Laos, Vietnam, and East Thailand, and Borneo Island. However, the phylogenic relationship of Vietnamese mouse deer has not been well characterized. In this study, we aimed to assess the genetic relationship of Vietnamese mouse deer by using cytochrome b sequences.

## Materials and Methods

### Sample collection

In this study, we collectedbone and tissue samples from 11 mouse deer. Nine samples (CT1 to CT9) were collected from Cat Tien National park(Dong NaiProvince) 2 samples (LD1-LD2) were collected from Lam Dong province. All collected samples were got permission from the local authorities and relatives.The samples were kept in −20˚C and transported to the laboratory. The remainders of sequences were derived from GenBank.

### DNA extraction

Total DNA were extracted from mouse deer samples using GeneJET Genomic DNA Purification Kit (K0721, Thermo scientific). Total DNA was resuspended with TE buffer and preserved at −20˚C.

### PCR

iProofHF Master Mix (1725310, Biorad) was applied for cytochrome b amplification, PCRs are performed in a final volume of 25 µl containing 2.5µlMaster Mix, 1 µl DNA template, 1 µl Forward and Reverse Primer, 20.5 µl distilled water. Primers of cytchome b amplification were as follows: F:5'-CCT CAR AAT GAT ATT TGKCCT CA-3', R:5'-CAG GMC TAT TCCTRG CHA TAC A-3'(Sarvani et al., 2018). PCR was performed under the following conditions: one cycle of DNA denaturation at 98$^{o}$C in 3 min; 40 cycles at 98$^{o}$C in 10 s; annealing at 50$^{o}$C in 30 s; extension at 72$^{o}$C in 30 s; final extension at 72$^{o}$C in 10 min. After PCR running, the gel electrophoresis of PCR product is performed on 1% agarose gel.

**Sequencing**

Amplified DNAs were purified using ExoSAP-IT PCR Clean up kit (Macrogen, Korea) and used as sequencing templates.The nucleotide sequences were determined using 3730XL DNA Analyzer (Macrogen, Korea). All PCR products are well-prepared, labeled and carefully packed for preventing the contamination among samples before sending out to nucleotide sequencing in Korea.

**Sequence analysis**

All Vietnamese mouse deer sequences (MW675310-MW675320) will be compared with the other groups derived from Genbank using MEGA6 program. The cytochrome b sequences will be aligned using CLUSTAL W.Tamura &Nei model which used as genetic distance model. Neighbor-joining method was used for phylogenetic tree construction. Bootstrap analyses (1000 replications) are appliedtoestimate the confidence in branching order.

**Results**

The results of PCR analysis of cytochrome b showed that 11 samples have specific bands from cytochrome b amplification (Figure 1), including 9 samples from Cat Tien National park (Dong NaiProvince) and 2 samples from Lam Dong Province.

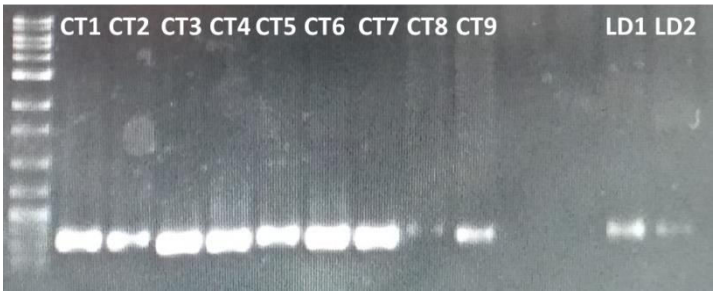


**Figure 1.**PCR analysis of cytochrome b in mouse deer. Eleven samples exposed specific bands of cytochrome bamplification. Samples CT1 to CT9 were collected from Cat Tien National park(Dong NaiProvince), samples LD1 and LD2 were collected from Lam Dong Province.

Analysis of cytochrome b sequences of 34 mouse deer (including 19 *Tragulusjavanicus*, 2 *Traguluskanchil*, 2 *Tragulusnapu*, 11 Vietnamese mouse deer samples) showed 37 polymorphic sites (Figure 2), representing 15.2% of total analyzed DNA sequence (243 bp). Vietnamese mouse deer exhibited 13 variable positions (14328, 14337, 14400, 14427, 14436, 14466, 14523, 14526, 14530, 14537, 14541, 14544, 14550), representing 5.3% of total analyzed DNA sequence. These new variable positions of Vietnamese mouse deer were found to be distinct to other mouse deer and they can be used for distinguishing Vietnamese mouse deer and others mouse deer.

As seen in Table 1, *Tragulusjavanicus* showed the highest within group distance (0.025 ± 0.008), while the lowest within group distance was observed in Vietnamese mouse deers (0.001 ± 0.000). The between group distance analysis showed that the distance between Vietnamese mouse deer and *Tragulusnapu* was highest (0.051 ± 0.022). The distance between Vietnamese mouse deer and *Traguluskanchil* was 0.035 ± 0.016. The lowest distance was noticed between Vietnamese

mouse deer and *Tragulusjavanicus* (0.031 ± 0.014). These results revealed that Vietnamese mouse deer have a close relationship to *Tragulusjavanicus* group.

The neighbor-joining tree was used to build the phylogenetic relationship of Vietnamese mouse deer and other mouse deer (Figure 3). The mouse deer were arranged into different clusters. The first cluster was *Tragulusnapu* with 99% bootstrap probability. *Traguluskanchil* was gathered to *Tragulusjavanicus* in one group with 93% bootstrap probability. Vietnamese mouse deers were clustered in distinct group with 100% bootstrap probability. Moreover, the variable position at 14325 (A → G) conduced to the separation of CT2, CT4, CT7, and CT8 in one group with 64% bootstrap probability

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                                1111111111 1111111111 1111111111 1111111
                                4444444444 4444444444 4444444444 4444444
                                3333333333 3344444444 4444455555 5555555
                                1222334589 9900023356 6678902222 3334445
                                7358176221 4706973640 3664900369 0571470
NC_020753.1_Tragulus_kanchil    CCAAACCACA CCACTCCTAG TGCCCTACTC CCCGACA
JN632709.1_Tragulus_kanchil     .......... .......... .......... .......
AB122112.1_Tragulus_napu        ....G..CTG TTGTCT.C.. .AT..C.ACA .T.....
AB122111.1_Tragulus_napu        .T..G..CTG TT.TCT.C.. .AT..C.AC. .TT....
AB122110.1_Tragulus_javanicus   TT....T... ......TC.A CA..T..ACA AT...T.
AB122109.1_Tragulus_javanicus   TT....T... ......TC.A CA.TT.GA.A AT...T.
AB122108.1_Tragulus_javanicus   TT....T... ......TC.A CA.TT.GA.A AT...T.
AB122107.1_Tragulus_javanicus   TT....T... ......TC.A CA.TT.GA.A AT...T.
AB122106.1_Tragulus_javanicus   TT....T... ......TC.A CA..T.GA.A AT.A.T.
AB122096.1_Tragulus_javanicus   .......... .......C.. .A..T..G.. .T.....
AB122105.1_Tragulus_javanicus   .......... .......... .......... .......
AB122104.1_Tragulus_javanicus   .......... .......... .......... .......
AB122103.1_Tragulus_javanicus   .......... .......... .......... .......
AB122102.1_Tragulus_javanicus   .......... .......... .......... ....G..
AB122101.1_Tragulus_javanicus   .......... .......... .......... .......
AB122100.1_Tragulus_javanicus   .......... .......... .......... .......
AB122099.1_Tragulus_javanicus   .......... .......... .......... .......
AB122098.1_Tragulus_javanicus   .......... .......... .......... .......
AB122097.1_Tragulus_javanicus   .T........ T......C.. .A.....A.. .T.....
AB122094.1_Tragulus_javanicus   .T........ T......C.. .A.....A.. .T.....
D32189.1_Tragulus_javanicus     .......... .......CG. .A...C.G.. .......
AB122093.1_Tragulus_javanicus   .......... .......C.. .A...C.G.. .......
AB122095.1_Tragulus_javanicus   .......... .......C.. .A...C.G.. .......
CT1                             ...T.T.... ..G..T.C.. .A.....GC. T.TAG.G
CT2                             ..GT.T.... ..G..T.C.. .A.....GC. T.TAG.G
CT3                             ...T.T.... ..G..T.C.. .A.....GC. T.TAG.G
CT4                             ..GT.T.... ..G..T.C.. .A.....GC. T.TAG.G
CT5                             ...T.T.... ..G..T.C.. .A.....GC. T.TAG.G
CT6                             ...T.T.... ..G..T.C.. .A.....GC. T.TAG.G
CT7                             ..GT.T.... ..G..T.C.. .A.....GC. T.TAG.G
CT8                             ..GT.T.... ..G..T.C.. .A.....GC. T.TAG.G
CT9                             ...T.T.... ..G..T.C.. .A.....GC. T.TAG.G
LD1                             ...T.T.... ..G..T.C.. .A.....GC. T.TAG.G
LD2                             ...T.T.... ..G..T.C.. .A.....GC. T.TAG.G
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**Figure 2.**Variable positions of the cytochrome b gene in mouse deer. Sequence identities are indicated by dots.

**Table 1**.Matrix of Tamura &Nei genetic distance among mouse deer. Lower triangular matrix values is mean genetic distances, upper triangular matrix values is standard errors.

| | Traguluskanchil | Tragulusnapu | Tragulusjavanicus | Vietnamese mouse deers |
|---|---|---|---|---|
| Traguluskanchil | | 0.020 | 0.002 | 0.016 |
| Tragulusnapu | 0.044 | | 0.017 | 0.022 |
| Tragulusjavanicus | 0.005 | 0.037 | | 0.014 |
| Vietnamese mouse deers | 0.035 | 0.051 | 0.031 | |

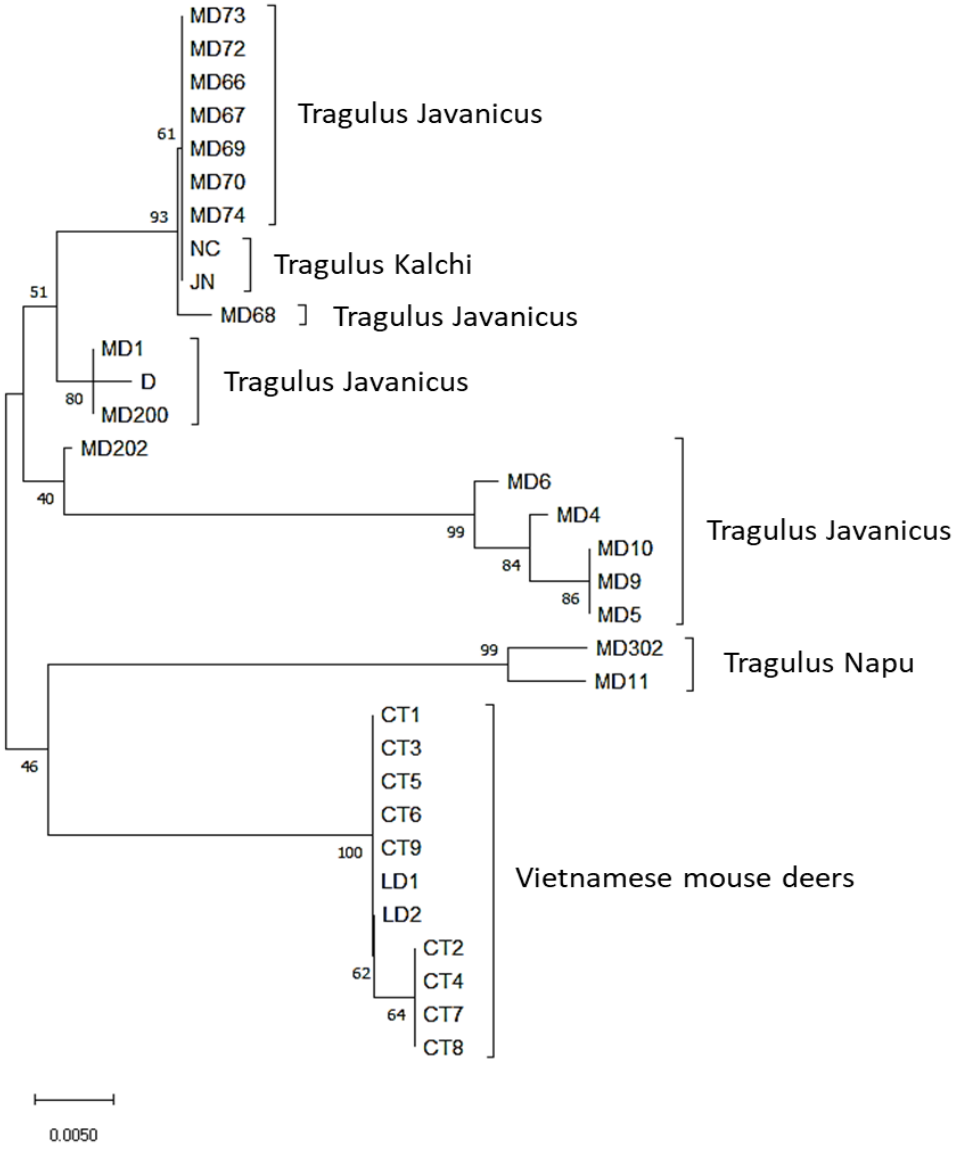


**Figure 3**.Phylogenetic tree constructed from cytochrome b sequences of mouse deer by the neighbor-joining method. Bootstrap resampling was done 1000 times, and resulting bootstrap values are shown on the corresponding branches.

## Discussion

Southeast Asia is a biodiversity hotspot (Long et al., 2014). *Tragulusjavanicus* and *Tragulusnapu* have been found to be distributed broadly across mainland southeast Asia (Corbet and Hill, 1992; Wilson and Reeder, 1993; Endo et al., 2004).Recently, the silver-backed chevrotain *Tragulusversicolor* has been showed to be still exists in Vietnam (Nguyen et al., 2019).Three main clusters were observed on phylogenetic trees, including*Tragulusjavanicus* from Malayan Peninsula and Laos, *Tragulusjavanicus* from Borneo Island, and *Tragulusnapu* from Borneo and PulauTioman (Endo et al., 2004). *Tragulusnapu* forms the sister lineage to all populations of *Tragulusjavanicus*, thereby supporting the current separation of these taxa at species level and traditional taxonomical identification of the two species by throat color.

In this investigation, Vietnamese mouse deer from the Southern of Vietnam have not been well genetically characterized. The analysis of variable position and phylogeny revealed the close relationship between Vietnamese mouse deer and *Tragulusjavanicus* group. The present work applied a molecular clock equation ($\lambda = d/2t$) to estimate divergence of mouse deer from Vietnam; where $\lambda$ = substitution rate/site/year, d = mean distance between individuals within groups or mean distance between groups and t = divergence time), the above distances and employing the substitution rate ($0.098 \times 10^{-6}$ substitution/site/year) for cytochrome b in mammalian species (Nabholz et al., 2009). The result showed that Vietnamese mouse deer exhibited the latest diverged from *Tragulusjavanicus* (~ 158000 YBP). Vietnamese mouse deer and *Traguluskanchil* showed the earlier divergence than *Tragulusnapu* (~ 260000YBP vs. 179000 YBP). This result suggested that Vietnamese mouse deer have a close relationship to *Tragulusjavanicus*.

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