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A record and phylogenetic analysis of *Muntiacus vuquangensis* in Vietnam assessed by cytochrome b

Ha Thanh Tung, Ha Thanh Thinh, Nguyen Thi Phuong Mai*
Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology, Da Lat, Vietnam

Abstract

This study aimed to assess the genetic relationship of giant Muntjac sampmles from Museum Biology – Tay Nguyen Institute for Scientific Research and other Muntjac samples from Genbank. PCR was applied to amplify the cytochrome b (cytB) gene of these sample. The PCR products were used as the templates for sequencing. The results showed that there were 26 nucleotide variation positions, compared with other species (accounting for about 6% of the analyzed cytB sequences). There was one variable position in samples of *Muntiacus vuquangensis* at position 284. The phylogenetic tree showed that the giant Muntjac sample in this study located with other *Muntiacus vuquangensis* with 100 % bootstrap value.

Keywords: cytochrome b, genetic relationship, giant muntiac

Introduction

The giant muntjac (*Muntiacus vuquangensis*) is one of the most endangered species in Vietnam. They were first recorded in 1994 in Vu Quang Nature Reserve, Ha Tinh province, North Central Vietnam (Tuoc *et al*., 1994) [2] and described in 1996 (Schaller and Vrba, 1996) [3]. The *Muntiacus* genus has 12 species, and there are 6 species in Vietnam, including *Muntiacus truongsonensis*, *Muntiacus vuquangensis*, *Muntiacus puhoatensis*, *Muntiacus rooseveltorum*, *Muntiacus muntjak*, *Muntiacus feae*. This proves that Vietnam is one of the remarkable centers of the distribution of *Muntiacus*. The taxonomy of *Muntiacus* based on morphology and molecular analysis have been reported by previous studies (Timmins *et al*., 1998; Timmins *et al*., 2008; Chen *et al*., 2008; Rasphone, 2010; Turvey *et al*., 2016; Stimpson *et al*., 2019) [4, 5, 6, 7, 8, 9].

The giant muntjac is mainly found in the Truong Son Mountains on the border of Vietnam and Laos, although they have also been found in the Mondulkiri province of Cambodia and in central Vietnam. (Bauer, 1997; Schaller and Vrba, 1996; Timmins, *et al*., 1998) [1, 3, 4]. In Vietnam, the giant muntjac has been recorded in several locations such as the South of Pu Mat Nature Reserve in Nghe An province and the Vu Quang Nature Reserve in Ha Tinh province (Dawson and Tuoc, 1997; Eve *et al*., 2000) [11]. Most evidence for the existence of giant muntjac is recorded by camera traps. In this study, giant muntjac samples were derived from Biological Museum – Tay Nguyen Institute for Scientific Research. Six samples were used for DNA extraction and amplification of cytB, then used as templates for sequencing. The genetic relationship was estimated by the alignment and genetic distance analysis of the samples in this study and other sequences from Genbank.

Materials and methods

Sample collection

Giant muntjac samples were supplied by the Museum Biology – Tay Nguyen Institute for Scientific Research. We obtained samples of 6 specimens (4 tissue samples, 2 bone samples) from the Museum Biology – Tay Nguyen Institute for Scientific Research. Bone samples were collected in small amounts (<5 mg) from parts of the giant muntjacs.

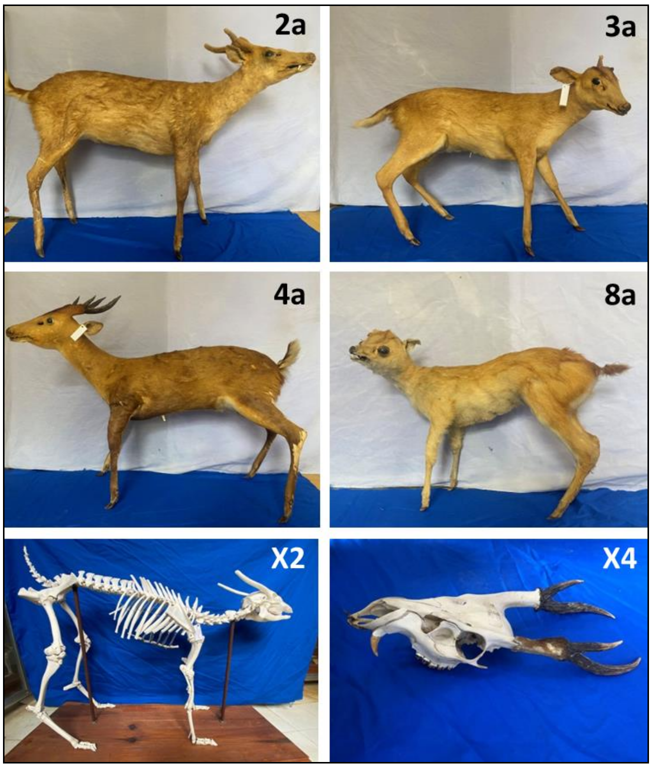


Fig 1: Pictures of giant muntjac obtained at Museum Biology- Tay Nguyen Institute for Scientific Research.

DNA extraction, amplification and sequencing

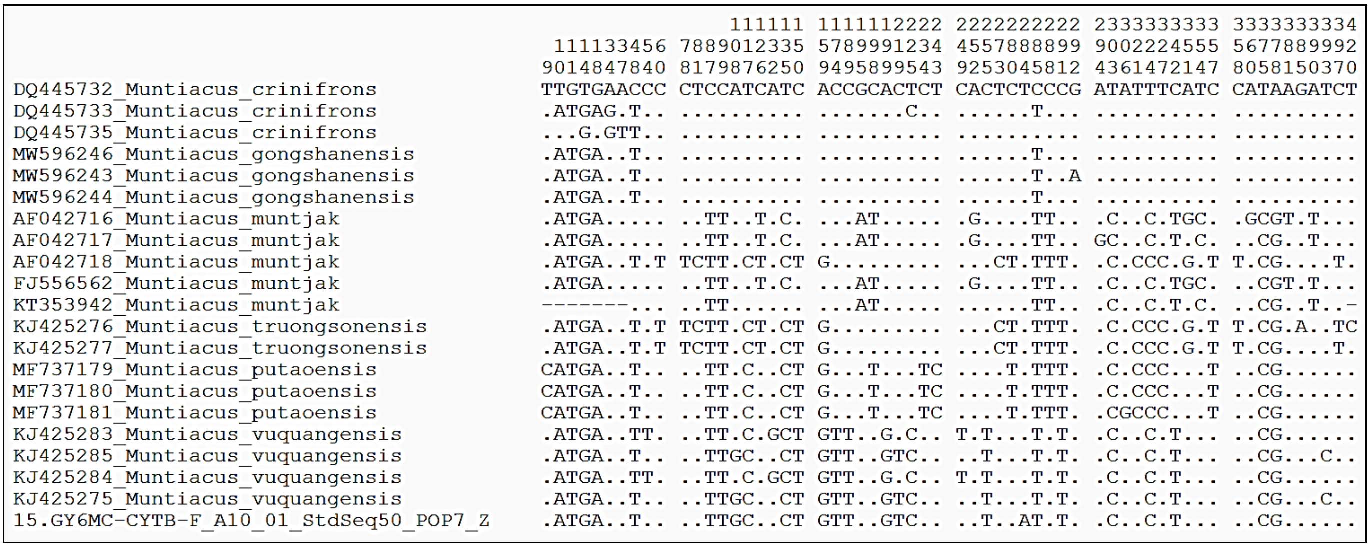
Total DNA of giant muntjac samples was extracted using GeneJET Genomic DNA Purification Kit (K0721, Thermo scientific). The extracted DNA were used as the templates to amplify the *Cyt b* fragment by iProof HF Master Mix kit (1725310, Biorad). Forward and reverse primers for *Cyt b* gene were: SoriF 5'-TGACATGAAAAATCATCGTTG-3' and SoriR 5'-CCATCTCTGGTTTACAAGAC-3'. PCR reactions are performed in a final volume of 25 µl containing 2.5µl Master Mix, 1 µl DNA template, 1 µl Forward and Reverse Primer, 20.5 µl distilled water. The thermal cycle is as follows: 94°C for 10 min; 70 cycles at 94°C for 45 s, 50°C for 45 s and 72°C for 45 s; final extension at 72°C for 10 min. PCR products were electrophoresed with 1% agarose gel. The PCR products were sent to Nam Khoa Company for sequencing.

Data analysis

Phylogenetic tree were built by Neighbour-joining method using MEGA version 6 (Tamura *et al*., 2013). The sequences of Vietnamese giant muntjacs were trimmed and compared with others from Genbank. CLUSTAL W was used to align the sequences (Tamura *et al*., 2011). The genetic distance was calculated by the Tamura and Nei model (Saitou *et al*., 1987). The confidence in the branching order was evaluated using a bootstrap analysis (using 1000 replications).

Results

In this study, the PCR product of cytB gene is 443bp, after sequencing, the noise region at both ends is removed, the remaining sequence fragment is analyzed with the size of 430bp. One of six samples showed a good result of sequencing, thus it was used for sequences analysis. The results of alignment of cytB sequences showed that there was 1 variable site compared to the same species (accounting for about 0.2% of the analyzed cytB sequence) at position 284. There were 26 nucleotide variation positions, compared with other species (accounting for about 6% of the analyzed cytB sequences) (Figure 2). Variations in these sequences were referred to as nucleotide substitutions, nucleotide insertions and deletions were not found in the sequence analysis. All samples analyzed had nucleotide variation positions of 10, 11, 14, 18, 48, 87, 99, 108, 117, 135, 150, 159, 174, 189, 199, 219, 225, 252, 284, 285, 291, 303, 324, 342, 375, 378.



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                                                          111111 1111112222 2222222222 2333333333 3333333334
                                           111133456 7889012335 5789991234 4557888899 9002224555 5677889992
                                          9014847840 8179876250 9495899543 9253045812 4361472147 8058150370
DQ445732_Muntiacus_crinifrons             TTGTGAACCC CTCCATCATC ACCGCACTCT CACTCTCCCG ATATTTCATC CATAAGATCT
DQ445733_Muntiacus_crinifrons             .ATGAG.T.. .......... .......C.. ......T... .......... ..........
DQ445735_Muntiacus_crinifrons             ...G.GTT.. .......... .......... .......... .......... ..........
MW596246_Muntiacus_gongshanensis          .ATGA..T.. .......... .......... ......T... .......... ..........
MW596243_Muntiacus_gongshanensis          .ATGA..T.. .......... .......... ......T..A .......... ..........
MW596244_Muntiacus_gongshanensis          .ATGA..T.. .......... .......... ......T... .......... ..........
AF042716_Muntiacus_muntjak                .ATGA..... ..TT..T.C. ...AT..... .G....TT.. .C..C.TGC. .GCGT.T...
AF042717_Muntiacus_muntjak                .ATGA..... ..TT..T.C. ...AT..... .G....TT.. GC..C.T.C. ..CG..T...
AF042718_Muntiacus_muntjak                .ATGA..T.T TCTT.CT.CT G......... ...CT.TTT. .C.CCC.G.T T.CG....T.
FJ556562_Muntiacus_muntjak                .ATGA..... ..TT..T.C. ...AT..... .G....TT.. .C..C.TGC. ..CGT.T...
KT353942_Muntiacus_muntjak                -------... ..TT...... ...AT..... ......TT.. .C..C.T.C. ..CG..T..-
KJ425276_Muntiacus_truongsonensis         .ATGA..T.T TCTT.CT.CT G......... ...CT.TTT. .C.CCC.G.T T.CG.A..TC
KJ425277_Muntiacus_truongsonensis         .ATGA..T.T TCTT.CT.CT G......... ...CT.TTT. .C.CCC.G.T T.CG....T.
MF737179_Muntiacus_putaoensis             CATGA..T.. ..TT.C..CT G...T...TC ....T.TTT. .C.CCC...T ..CG......
MF737180_Muntiacus_putaoensis             CATGA..T.. ..TT.C..CT G...T...TC ....T.TTT. .C.CCC...T ..CG......
MF737181_Muntiacus_putaoensis             CATGA..T.. ..TT.C..CT G...T...TC ....T.TTT. .CGCCC...T ..CG......
KJ425283_Muntiacus_vuquangensis           .ATGA..TT. ..TT.C.GCT GTT..G.C.. T.T...T.T. .C..C.T... ..CG......
KJ425285_Muntiacus_vuquangensis           .ATGA..T.. ..TTGC..CT GTT..GTC.. ..T...T.T. .C..C.T... ..CG...C..
KJ425284_Muntiacus_vuquangensis           .ATGA..TT. ..TT.C.GCT GTT..G.C.. T.T...T.T. .C..C.T... ..CG......
KJ425275_Muntiacus_vuquangensis           .ATGA..T.. ..TTGC..CT GTT..GTC.. ..T...T.T. .C..C.T... ..CG...C..
15.GY6MC-CYTB-F_A10_01_StdSeq50_POP7_Z    .ATGA..T.. ..TTGC..CT GTT..GTC.. ..T..AT.T. .C..C.T... ..CG......
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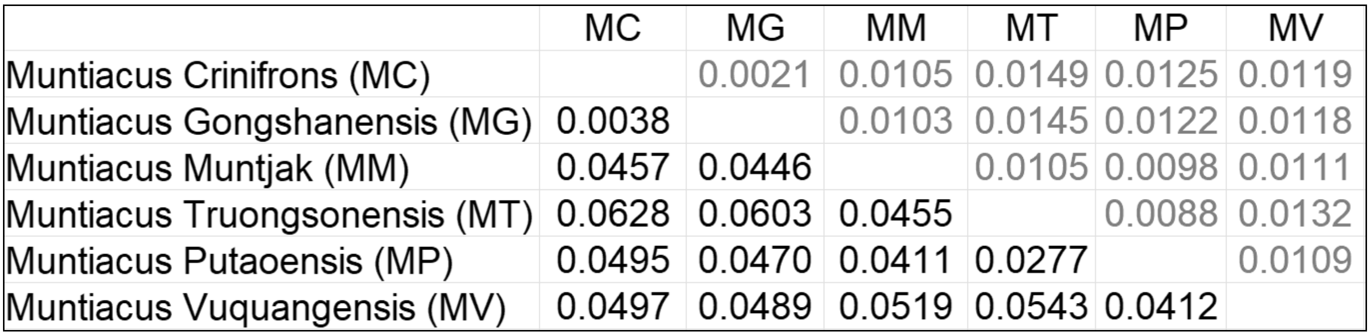
Fig 2: Polymorphic nucleotide positions in cytochrom b gene sequences from giant Muntjac samples. The dot (".") represents the similar nucleotide positions.

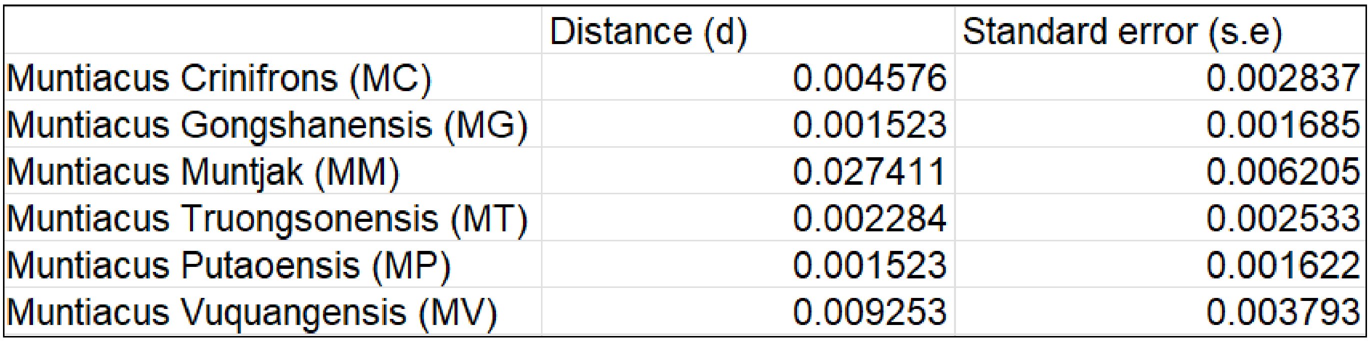
The analysis of genetic distance between groups based on cytB sequence showd that the highest distance the analyzed group and *Muntiacus Truongsonensis* groups (0.0543 ± 0.0132), while the distance with *Muntiacus Putaoensis* was lowest (0.0412 ± 0.0109) (Figure 3). The genetic distance within group of the *Muntiacus vuquangensis* was 0.009253 ± 0.003793 (Figure 4).

The phylogenic analysis of cyt B sequences of *Muntiacus vuquangensis* samples compared with other giant Muniac samples in the world was presented in Figure 5. The results showed that the giant Muntjac samples belonged to the group with *Muntiacus vuquangensis* with the bootstrap value reaching 100%.



	MC	MG	MM	MT	MP	MV
Muntiacus Crinifrons (MC)		0.0021	0.0105	0.0149	0.0125	0.0119
Muntiacus Gongshanensis (MG)	0.0038		0.0103	0.0145	0.0122	0.0118
Muntiacus Muntjak (MM)	0.0457	0.0446		0.0105	0.0098	0.0111
Muntiacus Truongsonensis (MT)	0.0628	0.0603	0.0455		0.0088	0.0132
Muntiacus Putaoensis (MP)	0.0495	0.0470	0.0411	0.0277		0.0109
Muntiacus Vuquangensis (MV)	0.0497	0.0489	0.0519	0.0543	0.0412	

Fig 3: Matrix of Tamura & Nei genetic distance between giant Muntjac samples. Lower triangular matrix values were mean genetic distance, Upper triangular matrix values were standard errors.



	Distance (d)	Standard error (s.e)
Muntiacus Crinifrons (MC)	0.004576	0.002837
Muntiacus Gongshanensis (MG)	0.001523	0.001685
Muntiacus Muntjak (MM)	0.027411	0.006205
Muntiacus Truongsonensis (MT)	0.002284	0.002533
Muntiacus Putaoensis (MP)	0.001523	0.001622
Muntiacus Vuquangensis (MV)	0.009253	0.003793

Fig 4: The matrix of genetic distance within testing groups

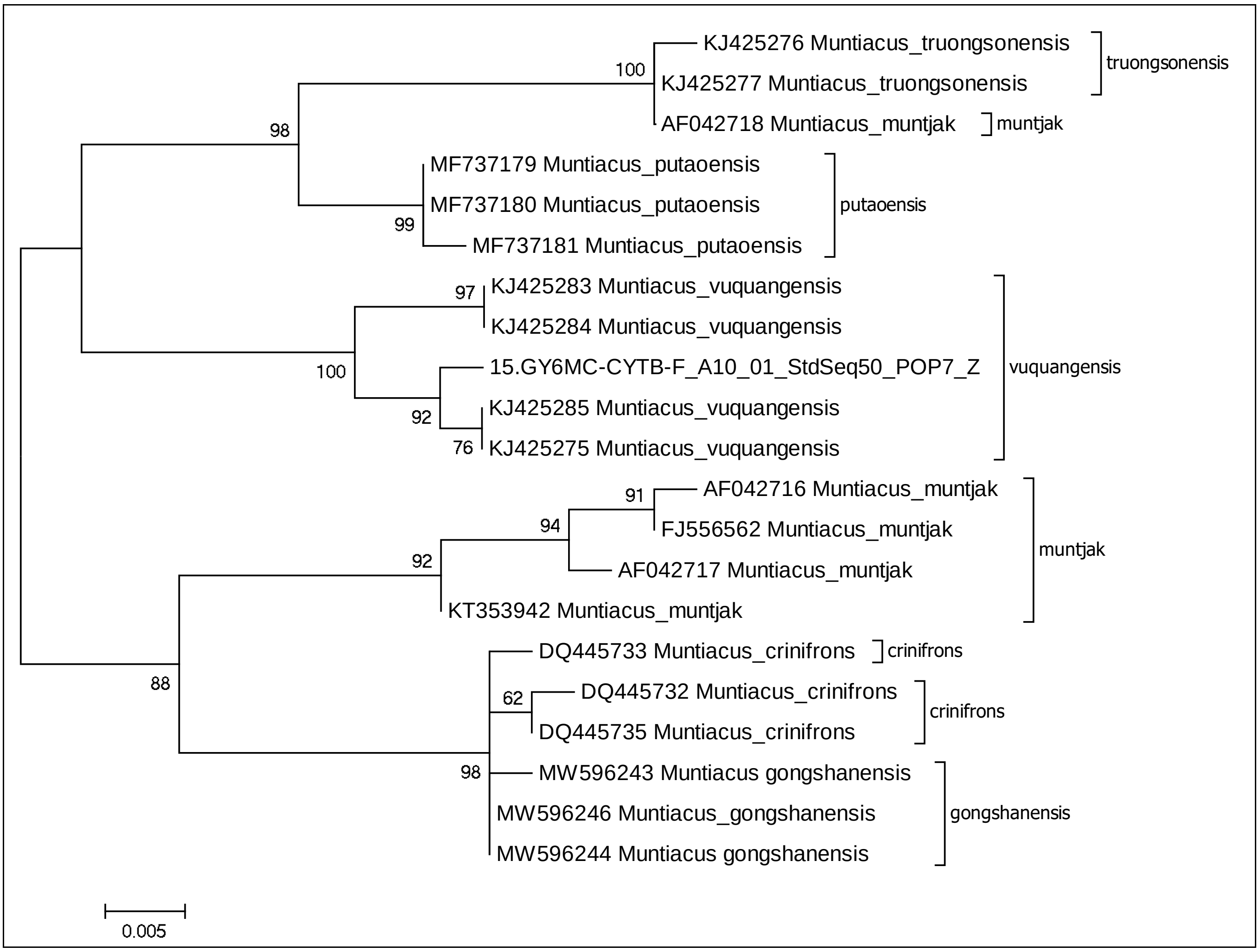


Fig 5: Phylogenetic tree constructed from cytB sequences of giant Muntjac by the neighbor-joining analysis method. Sample 2a is indicated by GY6M. Bootstrap resampling was done 1000 times, and resulting bootstrap values are shown on the corresponding branches.

Acknowledgements

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