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Original Article

Genetic similarities and phylogenetic analysis of Muntjac (*Muntiacus* spp.) by comparing the nucleotide sequence of 16S rRNA and cytochrome B genome

Semelhanças genéticas e análise filogenética de muntjac (*Muntiacus* spp.) comparando a sequência de nucleótidos de rRNA 16S e genoma citocromo B

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Abstract

This study aimed to identify the phylogenetic similarities among the muntjac (*Muntiacus spp.*). The phylogenetic similarities among seven major muntjac species were studied by comparing the nucleotide sequence of 16s rRNA and cytochrome b genome. Nucleotide sequences, retrieved from NCBI databases were aligned by using DNASTAR software. A phylogenetic tree was created for the selected species of muntjac by using the maximum likelihood method on MEGA7 software. The results of nucleotide sequences (16s rRNA) showed phylogenetic similarities between, the *M. truongsonensis* and *M.* rooseveltorum had the highest (99.2%) while the lowest similarities (96.8%) found between *M. crinifrons* and *M. putaoensi*. While the results of nucleotide sequences (Cty b) showed the highest similarity (100%) between *M. muntjak* and *M. truongsonensis* and the lowest s (91.5%) among *M. putaoensis and M. crinifrons*. The phylogenetic tree of muntjac species (16s rRNA gene) shows the main two clusters, the one including *M. putaoensis*, *M. truongsonensis*, *M.* rooseveltorum, and *M. muntjak,* and the second one including *M. crinifrons* and *M. vuquangensis.* The *M. reevesi* exists separately in the phylogenetic tree. The phylogenetic tree of muntjac species using cytochrome b genes shows that the *M. muntjak* and *M. truongsonensis* are clustered in the same group.

Keywords: barking deer, Muntjac, phylogenetic, 16S rRNA, Cty-b.

Resumo

Este estudo visou identificar as semelhanças filogenéticas entre os muntjac (*Muntiacus spp.*). As semelhanças filogenéticas entre sete grandes espécies muntjac foram estudadas comparando a sequência de nucleótidos de 16s rRNA e genoma citocromo b. As sequências de nucleótidos, obtidas a partir de bases de dados NCBI, foram alinhadas utilizando o software DNASTAR. Foi criada uma árvore filogenética para as espécies selecionadas de muntjac utilizando o método de probabilidade máxima no software MEGA7. Os resultados das sequências de nucleótidos (16s rRNA) mostraram semelhanças filogenéticas entre o *M. truongsonensis* e o *M. rooseveltorum* tiveram o maior número (99,2%) enquanto as semelhanças mais baixas (96,8%) encontradas entre *M. crinifrons* e *M. putaoensi*. Enquanto os resultados das sequências de nucleótidos (Cty-b) apresentaram a maior semelhança (100%) entre *M. muntjak* e *M. truongsonensis* e os mais baixos (91,5%) entre M. *putaoensis* e *M. crinifrons*. A árvore filogenética das espécies muntjac (gene rRNA 16s) mostra os dois principais aglomerados, o que inclui *M. putaoensis*, *M. truongsonensis*, *M. rooseveltorum* e *M. muntjak*, e o segundo incluindo *M. crinifrons* e *M. vuquangensis*. O *M. reevesi* existe separadamente na árvore filogenética. A árvore filogenética das espécies muntjac usando genes citocromo b mostra que os *M. muntjak* e M. truongsonensis estão agrupados no mesmo grupo.

Palavras-chave: veados ladrões, Muntjac, filogenético, 16S rRNA, Cty-b.

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1. Introduction

Muntjac is commonly known as deer and recognized for their beauty and grace. Male muntjac deer has bony antlers that annually molt while females lack antlers (Wikipidia, 2007a, b). Muntjac deer is belonging to the genus *Muntiacus,* family Cervidae, and order Artiodactyla and of the class Mammalia (Wikipidia, 2007a, b). They are classified into nine species, which are *Muntiacus feae*, *Muntiacus gongshanensis*, *Muntiacus crinifrons*, *Muntiacus reevesi*, *Muntiacus putaoensis*, and *Muntiacus rooseveltorum* (Giao et al., 1998; Wang and Lan, 2000; Shi and Cai-Xia, 1988; Nowak and Paradiso, 1991; Amato et al., 1999). The most primitive species of order Artiodactyla is the Barking deer and it is also known as the ancestor of the living families of artiodactyls (Dubost, 1971; Godina et al., 1962; Colbert, 1969; Barrette, 1977). The most strange fact about Barking deer is they contain the smallest number of diploid chromosomes (male contain seven and female contain six) yet described in any other mammal (Wurster and Benirschke, 1970).

The muntjac deer and their nine classified species are distributed in most regions of all around the world. They are distributed in tropical and subtropical deciduous forests, scrub forests, savannas, and grasslands. Their altitudes ranging from sea level to 3000 m and prefer the habitat of diverse woodland like large areas of woodland which are near to pasture land. They are found in Asia, Europe, northern Africa, and South and North America. They are native to South East Asia including India and Srilanka to Southern China, Bangladesh, Taiwan, Japan, and Indonesian islands (Wikipidia, 2007a, b). This study aimed to identify phylogenetic similarities among major muntjac species (*Muntiacus spp.*).

Generally, phylogenetic studies help to determine the relationships among genes or species, however, they can also give valuable insights into species' migration, changes in demographic patterns, and causes of viral infection (Yang and Rannala, 2012). In phylogenetic analysis, the genomic materials are used and achieved from chromosomal DNA which is present in the nucleus, and additional chromosomal DNA, present in eukaryotes organelles like chloroplasts and mitochondria (Abdoli et al., 2018). For the phylogenetic relationships concerning ancient divergences, sequences of complete mitochondrial DNA (mtDNA) and nuclear ribosomal rRNA gene, have been used (Zardoya and Meyer, 1996; Van de Peer and Wachter, 1997; Naylor and Brown, 1998; Mindell and Honeycutt, 1990; Abouheif et al., 1998; Zardoya et al., 1998). Single mtDNA genes were used most of the time during research studies to find out the population and taxonomic relationships (Tsigenopoulos and Berrebi, 2000; Rocha-Olivares et al., 1999, reviewed in Meyer, 1993; Lovejoy and Araújo, 2000). The mitochondrion organelle contains genetic information that can help in phylogenetic analyses (Moritz et al., 1987).

2. Material and Methods

The phylogenetic analyses of the seven major muntjac species including Indian muntjac (*Muntiacus muntjak*), Reeves's muntjac (*Muntiacus reevesi*), Hairy fronted muntjac (*Muntiacus crinifrons*), Leaf muntjac (*Muntiacus putaoensis*), Gaint muntjac (Muntiacus vuquangensis), Roosevelt's muntjac (Muntiacus rooseveltorum), and Troung Son muntjac (Muntiacus truongsonensis), were studied using 16s rRNA and cytochrome-b genomes.

2.1. Data description

Nucleotide sequence of cytochrome-b and 16s rRNA genes for seven muntjac species including Indian muntjac, *Muntiacus muntjak* (Cty-b = NCBI GeneBank AF042718.1, 1140 bp), (16s rRNA = NCBI GeneBank AF108039.1, 495bp); Reeves's muntjac, *Muntiacus reevesi* (Cty-b = NCBI GeneBank AF042718.1, 1140 bp), (16s rRNA = NCBI GeneBank AF108037.1, 495bp); Hairy fronted muntjac, *Muntiacus crinifrons* (Cty-b = NCBI GeneBank DQ445735.1, 1140 bp), (16s rRNA = NCBI GeneBank AF108035.1, 495bp); Leaf muntjac, *Muntiacus putaoensis* (Cty-b = NCBI GeneBank KJ425280.1, 1140 bp), (16s rRNA = NCBI GeneBank AF108032.1, 495bp); Gaint muntjac, *Muntiacus vuquangensis* (Cty-b = NCBI GeneBank KJ425285.1, 1,140 bp), (16s rRNA = NCBI GeneBank AF108034.1, 495bp); Roosevelt's muntjac, *Muntiacus rooseveltorum* (Cty-b = NCBI GeneBank KJ425282.1, 1140 bp), (16s rRNA = NCBI GeneBank AF108031.1, 495bp); Troung Son muntjac, *Muntiacus truongsonensis* (Cty-b = NCBI GeneBank KJ425277.1, 1140 bp), (16s rRNA = NCBI GeneBank AF108033.1, 495bp); and for out group Brown bear, *Ursus arctos* (Cty-b = NCBI Genebank HG008044.1, 1,140 bp) and Tibetan blue bear, *Ursus arctos pruinosus* (16s rRNA = NCBI Genebank MG131905.1, 495 bp) were retrived from NCBI databases.

2.2. Genetic similarities and Phylogenetic analysis

For all the nucleotide sequences (Cytochrome-b and 16s rRNA), the MegAlign module of the DNASTAR software (DNASTAR laser gene) were used for alignment. CLUSTAL W method was employed for the alignment of all the sequences. For multiple assessments, the CLUSTAL W algorithms were used. For similarities of nucleotide sequence, the section of sequence distance in DNASTAR was used. The residue substitutions windows in this software show a table to demonstrate several residue substitutions predicted to have occurred to give rise to the differences of sequence in the current alignment (Abdoli et al., 2018).

For selected species of muntjac, the phylogenetic tree was created using the maximum livelihood method on MEGA7 software (Kumar et al., 2016). The phylogenetic relationship between all the aligned sequences was demonstrated in the phylogenetic tree. The bootstraps consensus tree inferred from 1000 replicates.

3. Results

The consequences of nucleotide alignment analysis as similarities of sequences among 16s rRNA and cytochrome b of selected muntjac species (*Muntiacus muntjak*, *Muntiacus reevesi*, *Muntiacus crinifrons*, *Muntiacus putaoensis*, *Muntiacus vuquangensis*, *Muntiacus rooseveltorum*, *Muntiacus truongsonensis*) and out-group is *Ursus arctos* *pruinosus* and *Ursus arctos* shown in Table 1 and Table 2 respectively.

The similarities among nucleotide sequences (16s rRNA), the *Muntiacus truongsonensis,* and *Muntiacus* rooseveltorum had the highest similarities (99.2%) and the lowest similarities (96.8%) among *Muntiacus crinifrons* and *Muntiacus putaoensi*. The Muntiacus reevesi had 97.8% similarities with *Muntiacus crinifrons*, 98.6% with *Muntiacus muntjak*, 97.2% *Muntiacus putaoensis*, 98.2% with *Muntiacus vuquangensis*, 98.4% *Muntiacus* rooseveltorum, 98.4% with *Muntiacus truongsonensis.* The *Muntiacus crinifrons* had 97.6% similarities with *Muntiacus muntjak,* 96.8% *Muntiacus putaoensis*, 98.0% with *Muntiacus vuquangensis*, 97.8% *Muntiacus* rooseveltorum, 97.8% with *Muntiacus truongsonensis.* The *Muntiacus muntjak* had 97.8% similarities with *Muntiacus putaoensis*, 98.0% with *Muntiacus vuquangensis*, 98.6% *Muntiacus* rooseveltorum, 98.6% with *Muntiacus truongsonensis.* The *Muntiacus putaoensis* had 97.8% similarities with *Muntiacus vuquangensis*, 98.0% *Muntiacus* rooseveltorum, 98.4% with *Muntiacus truongsonensis.* The *Muntiacus vuquangensis* had 98.2% similarities with *Muntiacus* rooseveltorum and *Muntiacus truongsonensis.* The *Muntiacus* rooseveltorumand *Muntiacus truongsonensis* had the highest similarities 99.2% (as shown in Table 1).

While the similarities among nucleotide sequences (Cty b), the highest similarities (100%) between *Muntiacus muntjak* and *Muntiacus truongsonensis* and the lowest similarities (91.5%) among *Muntiacus putaoensis and Muntiacus crinifrons*. The *Muntiacus putaoensis* had 94.1% similarities with *Muntiacus vuquangensis*, 91.5% with *Muntiacus crinifrons,* 96.8% *Muntiacus muntjak*, 92.8% with *Muntiacus reevesi*, 97.2% *Muntiacus* rooseveltorum, 96.8% with *Muntiacus truongsonensis.* The *Muntiacus vuquangensis* had 92.1% similarities with *Muntiacus crinifrons,* 94.3% *Muntiacus muntjak*, 92.5% with *Muntiacus reevesi*, 94.3% *Muntiacus* rooseveltorum, 94.3% with *Muntiacus truongsonensis.* The *Muntiacus crinifrons* had 91.9% similarities with *Muntiacus muntjak*, 92.1% with *Muntiacus reevesi*, 92.5% *Muntiacus* rooseveltorum, 91.9% with *Muntiacus truongsonensis.* The *Muntiacus muntjak* had 92.3% similarities with *Muntiacus reevesi*, 97.0% *Muntiacus* rooseveltorum, 100.0% with *Muntiacus truongsonensis.* The *Muntiacus reevesi* had 93.2% similarities with *Muntiacus* rooseveltorum, 92.3% with *Muntiacus truongsonensis.* And the *Muntiacus* rooseveltorum had 97.0% similarities with *Muntiacus truongsonensis* (as shown in Table 2).

Table 1. Similarity and divergence percentage of the 16s rRNA of muntjac species.

Percentage identity:											
Divergence		1	2	3	4	5	6	7	8		
	1		97.8	98.6	97.2	98.2	98.4	98.4	85.5	1	*M. reevesi*
	2	1.9		97.6	96.8	98.0	97.8	97.8	84.4	2	*M. crinifrons*
	3	1.4	2.1		97.8	98.0	98.6	98.6	85.0	3	*M. muntjak*
	4	2.3	2.3	1.6		97.0	98.0	98.4	85.0	4	*M. putaoensis*
	5	1.8	1.6	2.1	2.5		98.2	98.2	85.5	5	*M. vuquangensis*
	6	1.6	1.9	1.4	1.4	1.9		99.2	84.8	6	*M.* rooseveltorum
	7	1.6	1.9	1.4	1.0	1.9	0.8		84.8	7	*M. truongsonensis*
	8	16.0	17.0	16.6	16.4	16.3	16.9	16.7		8	*Ursus arctos pruinosus*
		1	2	3	4	5	6	7	8		

Table 2. Similarity and divergence percentages of the cytochrome b of muntjac species.

Percentage identity											
Divergences		1	2	3	4	5	6	7	8		
	1		94.1	91.5	96.8	92.8	97.2	96.8	77.7	1	
	2	5.7		92.1	94.3	92.5	94.3	94.3	78.7	2	*M. putaoensis*
	3	8.7	8.5		91.9	92.1	92.5	91.9	77.2	3	*M. vuquangensis*
	4	2.7	6.0	8.7		92.3	97.0	100.0	78.2	4	*M. crinifrons*
	5	6.5	7.5	7.9	7.7		93.2	92.3	78.4	5	*M. muntjak*
	6	2.3	6.0	8.0	3.1	6.6		97.0	78.2	6	*M. reevesi*
	7	2.7	6.0	8.7	0.0	7.7	3.1		78.2	7	*M.* rooseveltorum
	8	26.4	25.6	27.8	26.3	25.3	26.4	26.4		8	*M. truongsonensis*
		1	2	3	4	5	6	7	8		*Ursus arctos*

The phylogenetic tree of muntjac species (16s rRNA gene) shows the main two clusters, the one including *Muntiacus putaoensis*, *Muntiacus truongsonensisMuntiacus* rooseveltorum, and *Muntiacus muntjak,* and the second one including *Muntiacus crinifrons* and *Muntiacus vuquangensis* (Figure 1). While using cytochrome b genes shows that the *Muntiacus muntjak* and *Muntiacus truongsonensis* are clustered in the same group (see Figure 2). The *Muntiacus reevesi* exist separately in the phylogenetic tree (see Figure 1). The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (16s rRNA = -990.44, Cyt-b = -3254.06) is shown. The percentage of the tree in which the associated taxa clustered together is shown next to the branches. İnitial tree for the heuristic search were obtained automatically by applying Neighbor Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with lengths measured in the number of substitutions per site. The analysis involved 8 nucleotide sequence. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 436 (16s rRNA) 1127 (Cty-b) and positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood

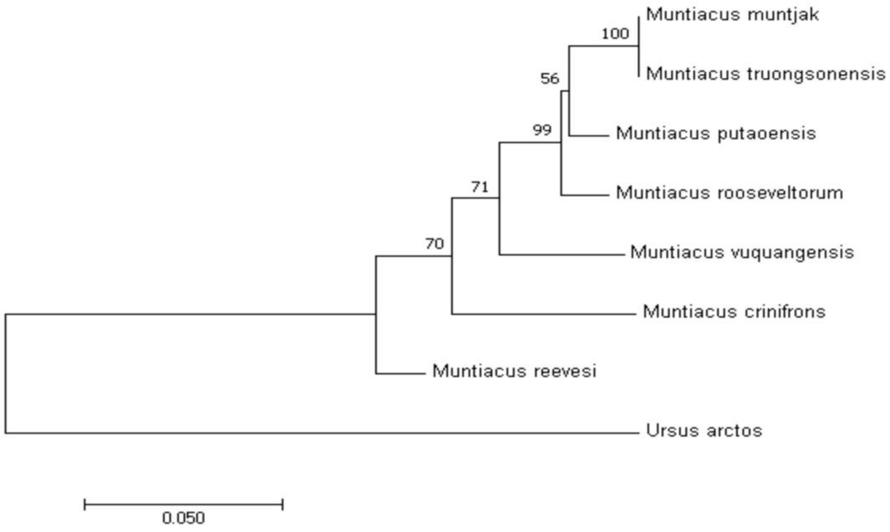

Figure 1. Molecular phylogenetic tree of Muntiacus species (16s rRNA gene) by maximum likelihood method.

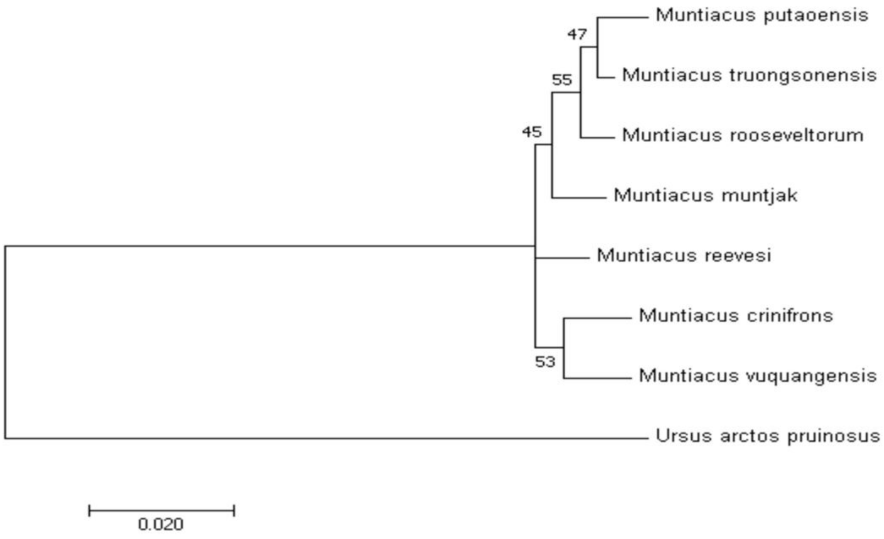

Figure 2. Molecular phylogenetic tree of Muntiacus species (Cty-b gene) by maximum likelihood method.

(-3254.06) is shown. The percentage of the tree in which the associated taxa clustered together is shown next to the branches. The initial tree for the heuristic search was obtained automatically by applying Neighbor Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with a superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 8 nucleotide sequence. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 1127 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

4. Discussion

The mitochondrial genome in animals contains 37 genes, of which, 2 are ribosomal RNA encoding genes while 13 genes encode respiratory chain proteins (Boore, 1999). The phylogenetic analyses of genus *Muntiacus* have long been studied (James et al., 2008; Timmins and Duckworth, 2016). The complete mitochondrial genome of *Muntiacus putaoensis* was similar to other species of muntjac deer (Wu and Fang, 2005; Shi et al., 2003; Zhang et al., 2004; Hassanin et al., 2012). In this study, the similarities among nucleotide sequences (16s rRNA genome), the *Muntiacus truongsonensis,* and *Muntiacus* rooseveltorum had the highest similarities (99.2%), and the lowest similarities (96.8%) among *Muntiacus crinifrons* and *Muntiacus putaoensi*. The phylogenetic tree of muntjac species (16s rRNA gene) shows the main two clusters, the one including *Muntiacus putaoensis*, *Muntiacus truongsonensis Muntiacus* rooseveltorum, and *Muntiacus muntjak,* and the second one including *Muntiacus crinifrons* and *Muntiacus vuquangensis.* These results reconfirm the results of James et al. (2008) using the 16s rRNA genome, where they indicated that the *Muntiacus putaoensis* formed a nested clade with *Muntiacus truongsonensis and Muntiacus* rooseveltorum due to minor variation in their sequences. Amato et al. (1999) also confirmed these results based on collective analysis of 4 gene regions, showed that the *Muntiacus putaoensis* is a sister taxon of the *Muntiacus truongsonensis and Muntiacus* rooseveltorum.

The phylogenetic analysis of five species of the Muntiacus genus using cytochrome b genes was studied by Li et al. (2017). The Cytochrome b genes of Muntiacus spp. (Including *Muntiacus putaoensis*, *Muntiacus truongsonensis, Muntiacus* rooseveltorum, and *Muntiacus vuquangensis*) were also discussed by Le et al. (2014). James et al. (2008) also used the Cty b gene of five species of Muntiacinae subfamily. The phylogenetic tree in this study of muntjac species using cytochrome b genes shows that the *Muntiacus muntjak* and *Muntiacus truongsonensis* are clustered in the same group and are closely related having 100% similarities in their sequences. The result contradicts the results of Li et al. (2017), which indicates that *Muntiacus truongsonensis* was closely related to *Muntiacus putaoensis* based on cytochrome b genome sequences.

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