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Phylogenetic Study of Complete Cytochrome *b* Genes in Musk Deer (Genus *Moschus*) Using Museum Samples

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As an endangered animal group, musk deer (genus *Moschus*) are not only a great concern of wildlife conservation, but also of special interest to evolutionary studies due to long-standing arguments on the taxonomic and phylogenetic associations in this group. Using museum samples, we sequenced complete mitochondrial cytochrome *b* genes (1140 bp) of all suggested species of musk deer in order to reconstruct their phylogenetic history through molecular information. Our results showed that the cytochrome *b* gene tree is rather robust and concurred for all the algorithms employed (parsimony, maximum likelihood, and distance methods). Further, the relative rate test indicated a constant sequence substitution rate among all the species, permitting the dating of divergence events by molecular clock. According to the molecular topology, *M. moschiferus* branched off the earliest from a common ancestor of musk deer (about 700,000 years ago); then followed the bifurcation forming the *M. berezovskii* lineage and the lineage clustering *M. fuscus*, *M. chrysogaster*, and *M. leucogaster* (around 370,000 years before present). Interestingly, the most recent speciation event in musk deer happened rather recently (140,000 years ago), which might have resulted from the diversified habitats and geographic barriers in southwest China caused by gigantic movements of the Qinghai–Tibetan Plateau in history. Combining the data of current distributions, fossil records, and molecular data of this study, we suggest that the historical dispersion of musk deer might be from north to south in China. Additionally, in our further analyses involving other pecora species, musk deer was strongly supported as a monophyletic group and a valid family in Artiodactyla, closely related to Cervidae. © 1999 Academic Press

Key Words: **musk deer; mitochondrial DNA; cytochrome *b* gene; molecular phylogeny**

INTRODUCTION

Mitochondrial DNA (mtDNA) are valuable molecules for the reconstruction of evolutionary relationships among populations, species, and higher taxa (Avis, 1986; Moritz *et al.*, 1987; Harrison, 1989; Hillis and Moritz, 1990). The cytochrome *b* (Cytb) gene is one of the best known of the 9–10 genes involved in the mitochondrial oxidative phosphorylation system (Hatefi, 1985). To date, many phylogenetic questions have been addressed based on Cytb sequences, and both the merits and the demerits of Cytb as a genetic marker have been discussed. Technically, with the advent and rapid development of the polymerase chain reaction (PCR)-based techniques, researchers can now recover genetic information from degraded specimens, such as bones, dried skins, excrement, and even fossils (Higuchi, 1988; Pääbo, 1989). This technical development has greatly enriched the possibilities of sampling, not only noninvasively from live animals, but also from museum specimens.

Musk deer (genus *Moschus*) are widely distributed in China and adjacent areas (especially the Qinghai–Tibetan Plateau and Himalayan areas) (Groves *et al.*, 1995). Many morphological studies have been done on the taxonomy of this group, but controversies concerning the numbers of species and subspecies and the phylogenetic relationships among them still remain (Ellerman, 1950; Gao, 1963, 1985; Li, 1981; Grubb, 1982; Groves *et al.*, 1986, 1995; Ohtaishi *et al.*, 1990; Sheng, 1989; Wang *et al.*, 1993). Based on the characteristics of external and skull morphology used in a multivariate analysis, Groves *et al.* (1995) suggested that there are five species of musk deer. They are Siberian musk deer (*M. moschiferus*), forest musk deer (*M. berezovskii*), black musk deer (*M. fuscus*), Alpine musk deer (*M. chrysogaster*), and Himalayan musk deer (*M. leucogaster*). Cytogenetically, Shi and Ma (1986) studied the mitotic and synaptomenal karyotypes of the forest musk deer (*M. berezovskii*). The diploid number was found to be 58, which concurred with the report on *M. moschiferus* by Sokolov *et al.*

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(1980). However, phylogenetic studies using molecular approaches have been rare. In this study, using museum samples, we sequenced complete Cytb genes of musk deer in order to provide molecular evidence for the unsolved questions in musk deer phylogeny.

MATERIALS AND METHODS

Sample Collection

Samples were acquired from collections of the Kunming Institute of Zoology, the Institute of Zoology in Beijing, and the Hei-long-jiang Natural Resources Institute in Harbin of China. All samples were pieces of dried skins cut from whole leather specimens stored for periods ranging from several years to decades (Table 1). All specimens were studied and taxonomically identified by morphologists based on analyses of the whole animals. In this study, a total of 15 individual samples was obtained, and 8 samples were found to have recoverable DNA representing all suggested species and one subspecies of *M. berezovskii* (see Table 1 for details).

DNA Extraction

We followed the method of Walsh *et al.* (1991) in our DNA extraction with a few optimizations for dried skin samples. All chemicals and utensils were sterilized before use. The DNA extraction was processed under ultraviolet-cleaned conditions. During the extraction, a negative control tube was prepared to monitor possible contamination. However, it deserves mention that in our experience, the success of DNA recovery from dried skin samples depends largely on the original condition of specimens. The hard parts of the specimens are generally less degraded and have proved to be DNA recoverable, while the soft or loose parts usually do not contain recoverable DNA due to overtreatment for antiseptic purposes or overdegradation in storage.

TABLE 1

Provenance of Museum Samples Used in this Study

Specimen	Location	Date sampled (no.)	Origin
M. moschiferus 1 (Siberian musk deer)	HNI	1979(/)	Yichun, Hei-longjiang Prov.
M. moschiferus 2 (Siberian musk deer)	HNI	1981(/)	Xiaoxinganling Mountain
M. chrysogaster.si-fanicus (Alpine musk deer)	KIZ	1982(82042)	Dege, Sichuan Prov.
M. fuscus (Black musk deer)	KIZ	1978(780414)	Bijiang, Yunnan Prov.
M. leucogaster (Himalayan musk deer)	BIZ	/ (T129)	Tibet
M. berezovskii bijiangensis-1 (Forest musk deer)	KIZ	1978 (780422)	Bijiang, Yunnan, Prov.
M. berezovskii bijiangensis-2 (Forest musk deer)	KIZ	1990 (R90139)	Tengchong, Yunnan Prov.
M. berezovskii cao-bangis (Forest musk deer)	KIZ	/ (006734)	Mile, Yunnan Prov.

Note. HNI, Hei-long-jiang Natural Resources Institute of China; KIZ, Kunming Institute of Zoology, the Chinese Academy of Sciences; BIZ, Institute of Zoology (Beijing), the Chinese Academy of Sciences.

TABLE 2

The 16 Primers Used in PCR and Sequencing of this Work

1.	L14724	5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′
2.	L14841	5′-CCATCCAACATCTCAGCATGATGAAA-3′
3.	L15026	5′-GGAGCATCAATATTCTTTATCTGCC-3′
4.	L15158	5′-GGATATGTCCTACCTTGAGGACAAA-3′
5.	L15267	5′-GGCTTCTCAGTAGACAAAGCAA-3′
6.	L15402	5′-CATCGGACGCAGACAAAATCCC-3′
7.	L15579	5′-CCCGAATGATACTTCCTATTTGCATA-3′
8.	L15738	5′-GCCTATTTTGAATTTTAGTAGCAGA-3′
9.	H14898	5′-TTGTATCGGATGTATAGTGTATTGCTA-3′
10.	H15042	5′-CTGCTCCGGATATGATGCCTAGTA-3′
11.	H15168	5′-GGTTGGTGATGACTGTTGCTCCTC-3′
12.	H15275	5′-GGATGAAGTGGAAGGCAAAGAATCG-3′
13.	H15413	5′-CCTAGAATGTCTTTGATGGTGTAGTA-3′
14.	H15605	5′-GGCTAGTACTCCTCCTAGTTT-3′
15.	H15749	5′-CTGGTTGTCCTCCAATTCATGTGAG-3′
16.	H15915	5′-AACTGCAGTCATCTCCGGTTTACAAGAC-3′

PCR and DNA Sequencing

We designed a group of primers to amplify the whole Cytb gene from the degraded DNA samples (Table 2). The reference sequences for primer design were from nine deer species (unpublished data). The universal primers of L14724, H15149, and L14841 were also used (Irwin *et al.*, 1991). Principally, each pair of primers was designed to cover a 200- to 300-bp fragment because for degraded DNA samples longer fragments usually cannot be amplified through PCR. Each entire Cytb sequence was generated by aligning and overlapping the eight fragment sequences. PCRs were done on a Robocycler (Stratagene). The PCR conditions were: predenature at 94°C for 2 min, then cycling at 94°C (30 s), 42–50°C (30 s, varying among primers), 72°C (1 min) for 40 cycles, and final extension at 72°C for 5 min. PCR products were purified through LMP agarose electrophoresis and roughly quantified by eye through EB staining.

For sequencing, an automatic DNA sequencer (ABI Model 377) was used for direct sequencing of double-stranded PCR products. A cycle sequencing protocol with FS kit (ABI) was used following instructions of the producer. All amplified fragments were sequenced with both light- and heavy-stranded primers.

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                                                                                                                    60
M.moschiferus       ATG ACC AAT ATC CGA AAA TCC CAC CCA TTA ATA AAA ATT GTA AAC AAT GCA TTC ATC GAC
M.chrysogaster      ... ... ... ..T ... ... ..T ... ... ... ... ..G ... ..G ... ..C ... ... ..T ..T
M.fuscus            ... ... ... ..T ... ... ..T ... ... ... ... ..G ... ..G ... ... ... ... ..T ..T
M.leucogaster       ... ... ... ..T ... ... ..T ... ... C.. ... ... ... ..G ... ... ... ... ... ..T
M.b.bijiangensis-1  ... ... ... ..T ... ... ..T ... ... ... ... ..G ... ..G ... ... ... ... ..T ..T
M.b.bijiangensis-2  ... ... ... ..T ... ... ..T ... ... ... ... ..G ... ..G ... ... ... ... ..T ..T
M.b.caobangis       ... ... ... ..T ... ... ..T ... ... ... ... ..G ... ..G ... ... ... ... ..T ..T
A.alces             ... ... ..C ... ... ... A.. ... ... ... ... ... ... ... ..T ..C ... ..T ..T ..T
                                                                                                                   120
M.moschiferus       CTC CCA GCC CCA TCA AAC ATC TCA TCC TGA TGA AAT TTC GGC TCC CTA CTA GGC ATC TGC
M.chrysogaster      ..T ... ... ... ... ... ... ... ... ... ... ..C ..T ... ... ... ... ... ... ...
M.fuscus            ... ... ... ... ... ... ... ... ... ... ... ..C ..T ... ... ... ... ... ... ...
M.leucogaster       ... ... ... ... ... ... ... ... ... ... ... ..C ..T ... ... ... ... ... ... ...
M.b.bijiangensis-1  ... ... ..T ... ... ... ... ... ... ... ... ... ..T ... ... ... ..G ... ... ...
M.b.bijiangensis-2  ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ...
M.b.caobangis       ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ...
A.alces             ..A ... ... ... ... ..T ... ... ..A ... ... ... ..G ..T ... ... T.. ..A G.T ...
                                                                                                                   180
M.moschiferus       CTA ATC CTT CAA ATC CTA ACA GGC CTA TTC CTA GCA ATA CAT TAC ACC TCT GAC ACA ATA
M.chrysogaster      ... ... A.C ... ... ... ... ... ... ..T ... ... ... ... ... ... ..T ... ...
M.fuscus            ... ... A.C ... ... ... ... ... ... ..T ... ... ... ... ... ... ..T ... ...
M.leucogaster       ... ..T A.C ... ... ... ... ... ... ..T ... ... ... ..C ... ... ... ..T ... ...
M.b.bijiangensis-1  ... ..T A.C ... ... ... ... ... ... ..T ... ... ... ... ..T ... ... ..T ... ...
M.b.bijiangensis-2  ... ..T A.C ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ..T ... ...
M.b.caobangis       ... ..T A.C ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ..T ... ...
A.alces             T.. ... T.A ... ... ..T ... ..A ... ... ... ... ... ... ..T ..A ... ..T ... ...
                                                                                                                   240
M.moschiferus       ACA GCA TTT TCC TCT GTT ACT CAC ATT TGC CGA GAC GTT AAC TAT GGC TGA ATT ATT CGA
M.chrysogaster      ... ... ... ... ..C ..C ... ... ... ... ... ... ... ..T ... ... ... ... ... ...
M.fuscus            ... ... ... ... ..C ..C ... ... ... ... ... ... ... ..T ..C ... ... ... ... ...
M.leucogaster       ... ... ... ... ..C ..C ... ... ... ... ... ... ... ..T ..C ... ... ... ... ...
M.b.bijiangensis-1  ... ... ... ... ..C ... ... ... ... ... ... ..T ... ... ..C ... ... ... ... ...
M.b.bijiangensis-2  ... ... ... ... ..C ... ... ... ... ... ... ..T ... ..T ..C ... ... ... ... ...
M.b.caobangis       ... ... ... ... ..C ... ... ... ... ... ... ..T ... ..T ..C ... ... ... ... ...
A.alces             ... ... ..C ... ... ..C ..C ..T ..C ... ... ..T ..A ..T ..C ... ... ..C ... ...
                                                                                                                   300
M.moschiferus       TAC ATA CAT GCA AAT GGA GCA TCA ATA TTC TTT ATC TGC CTG TTT ATA CAT GTA GGA CGA
M.chrysogaster      ..T ... ..C ... ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ...
M.fuscus            ..T ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.leucogaster       ..T ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.b.bijiangensis-1  ..T ... ..C ... ... ..G ... ... ... ... ... ... ... ... ..C ... ... ... ... ...
M.b.bijiangensis-2  ..T ... ..C ... ... ..G ... ... ... ... ... ... ... ... ..C ... ... ... ... ...
M.b.caobangis       ..T ... ..C ... ... ..G ... ... ... ... ... ... ... ... ..C ... ... ... ... ...
A.alces             ..T ..G ... ... ..C ... ..C ... ... ..T ..C ... ... T.A ... ... ... ... ... ...
                                                                                                                   360
M.moschiferus       GGC TTA TAC TAC GGA TCA TAC ACA TTC CTA GAA ACA TGA AAC ATC GGA GTC ATC CTC CTA
M.chrysogaster      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.fuscus            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.leucogaster       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.b.bijiangensis-1  ... ... ... ..T ... ... ... ... ..T ... ..G ... ... ... ... ... ... ... ... T..
M.b.bijiangensis-2  ... ... ... ..T ... ... ... ... ..T ... ..G ... ... ... ... ... ... ... ... T..
M.b.caobangis       ... ... ... ..T ... ... ... ... ..T ... ..G ... ... ... ... ... ... ... ... ...
A.alces             ..A C.. ... ... ... ..C ..T ..T ..T ... ... ... ... ... ... ... ..G ... ..T ...
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FIG. 1. Complete sequences of mitochondrial cytochrome *b* gene of musk deer.

DNA Sequence Alignment and Phylogenetic Analysis

DNA sequence alignments were done by eye; the variant sites were double checked by viewing the four-colored electromorph of sequencing results. For phylogenetic analysis, we used three mainstream algorithms for phylogenetic reconstruction, the most parsimony method using PAUP 3.0 (Swofford, 1989), the maximum likelihood method using Phylip 3.5c (Felsenstein, 1993), and the neighbor-joining (NJ) method using Mega 1.02 (Kumar *et al.*, 1993). Confidence values for internal lineages in parsimony analysis were assessed by bootstrapping (Felsnestein, 1985), and branch length confidence levels (CP values) for NJ trees were obtained through *t* tests. In order to root the trees, a set of available pecora sequences was tried as outgroup data. The relative rate tests were done following Sarich and Wilson (1973). The moose (*Alces alces*) was used as outgroup in the analysis.

In order to determine the taxonomic status of musk deer and its phylogenetic relationships with other

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                                                                                                           420
M.moschiferus          TTC ACA GTA ATA GCC ACA GCA TTC ATA GGA TAT GTC CTA CCT TGA GGA CAA ATA TCT TTC
M.chrysogaster         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.fuscus               ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.leucogaster          ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ...
M.b.bijiangensis-1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.b.bijiangensis-2     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.b.caobangis          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
A.alces                ..T ... ... ... ... ... ... ... G.T ... ... ... ... ..A ... ... ..G ... ..A ...
                                                                                                           480
M.moschiferus          TGA GGA GCA ACA GTC ATC ACT AAC CTT CTC TCA GCA ATT CCC TAC ATT GGT ACT AAC CTG
M.chrysogaster         ... ... ... ... ..T ..T ..C ..T ... ... ... ... ... ..A ... ... ... ... ... ...
M.fuscus               ... ... ..G ... ..T ..T ..C ..T ... ... ... ... ... ..A ... ... ... ... ..T ...
M.leucogaster          ... ... ... ... ..T ..T ..C ..T ... ... ... ... ... ..A ... ... ... ... ..T ...
M.b.bijiangensis-1     ... ... ... ... ..T ..T ..C ..T ... ... ... ... ... ..T ... ... ... ... ..T ...
M.b.bijiangensis-2     ... ... ... ... ..T ..T ..C ..T ... ... ... ... ... ..T ... ... ... ... ..T ...
M.b.caobangis          ... ... ... ... ..T ..T ..C ..T ... ... ... ... ... ..T ... ... ... ... ..T ..A
A.alces                ... ..G ... ... ... ..T ... ... ..C ..T ... ... ... ..A ... ... ... ... ..T ..A
                                                                                                           540
M.moschiferus          GTT GAG TGA ATT TGA GGA GGC TTC TCA GTA GAC AAA GCA ACA CTC ACC CGA TTC TTT GCC
M.chrysogaster         ... ..A ... ... ... ... ..T ... ... ... ... ... ... ... ... ..T ... ... ... ...
M.fuscus               ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ...
M.leucogaster          ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.b.bijiangensis-1     ... ..A ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.b.bijiangensis-2     ... ..A ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M.b.caobangis          ... ..A ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
A.alces                ... ..A ... ... ... ..C ..T ..T ... ... ... ... ... ..T ..A ... ... ..T ..C ...
                                                                                                           600
M.moschiferus          TTT CAC TTT ATC CTC CCA TTT ATC ATT GCA GCA CTC GCC ATG GTT CAT CTA CTC TTT CTC
M.chrysogaster         ..C ... ..C ..T ... ... ... ... ..C ... ... ... ..T ... ... ..C ... ... ... ...
M.fuscus               ... ... ..C ..T ... ... ... ... ..C ... ... ... ..T ... ... ..C ... ... ... ...
M.leucogaster          ..C ... ..C ..T ... ... ... ... ..C ... ... ... ..T ... ... ..C ... ... ... ...
M.b.bijiangensis-1     ..C ... ..C ... ... ... ... ... ..C ... ... ... ..T ... ... ..C ... ... ... ...
M.b.bijiangensis-2     ..C ... ..C ... ... ... ... ... ..C ... ... ... ..T ... ... ..C ... ... ... ...
M.b.caobangis          ..C ... ..C ... ... ..G ... ... ..C ... ... ... ..T ... ... ..C ... ... ... ...
A.alces                ..C ... ... ..T ... ... ... ... ..C ... ... ..T ... ..A ..C ..C T.. ..T ..C ...
                                                                                                           660
M.moschiferus          CAT GAA ACA GGA TCC AAT AAC CCA ACA GGA ATC ACA TCA GAC ATA GAC AAA ATC CCA TTT
M.chrysogaster         ..C ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ..C
M.fuscus               ..C ... ... ... ... ..C ... ... ... ... ... ... ... ..T ... ... ... ... ... ..C
M.leucogaster          ..C ... ... ... ... ..C ... ... ... ... ... ... ... ..T ... ... ... ... ... ..C
M.b.bijiangensis-1     ..C ... ... ... ... ..C ... ... ... ... ... ... ... ..T ... ... ... ... ... ..C
M.b.bijiangensis-2     ..C ... ... ... ... ..C ... ... ... ... ... ... ... ..T ... ... ... ... ... ..C
M.b.caobangis          ..C ... ... ... ... ..C ... ... ... ... ... ... ... ..T ... ... ... ... ... ..C
A.alces                ..C ... ... ... ... ..C ... ... ... ... ..T C.. ... ... GC. ... ... ... ... ...
                                                                                                           720
M.moschiferus          CAC CCC TAC TAC ACC ATC AAA GAT ATT CTA GGT ATC CTA TTA CTA ATC TTA ATC TTA ATA
M.chrysogaster         ... ... ... ... ... ... ... ..C ... ... ... G.. ... ... ... ... C.. G.. ... ...
M.fuscus               ... ... ... ... ... ... ... ..C ... ... ... G.. ... ... ... ... ... G.. ... ...
M.leucogaster          ... ... ... ... ... ... ... ..C ... ... ... G.. ... ... ... ... ... G.. ... ...
M.b.bijiangensis-1     ... ... ... ... ... ... ... ..C ... ... ... G.. ... A.. ... ... ... G.. ... ...
M.b.bijiangensis-2     ... ... ... ... ..T ... ... ..C ... ... ... G.. ... A.. ... ... ... G.. ... ...
M.b.caobangis          ... ... ... ... ... ... ... ..C ... ... ... G.. ... A.. ... ... ... G.. ... ...
A.alces                ... ..T ... ... ..T ... ... ... ..C T.. ... GC. ... C.C T.. .CT C.T T.. C.. ...
                                                                                                           780
M.moschiferus          GCA CTA GTG CTA TTT ACA CCC GAC CTA CTT GGA GAT CCG GAC AAC TAT ACT CCA GCA AAC
M.chrysogaster         A.. ... ..A ... ..C ... ..T ..T T.. ... ... ..C ... ... ..T ... ..C ..G ... ...
M.fuscus               A.. ... ..A ... ..C ... ..T ..T T.. ... ... ..C ... ... ..T ... ..C ... ... ...
M.leucogaster          A.. ... ..A ... ..C ... ..T ..T T.. ... ... ..C ... ... ..T ... ..C ... ... ...
M.b.bijiangensis-1     .T. ... ..A ... ..C ... ... ..T T.. ... ... ..C ... ... ..T ... ..C ... ... ...
M.b.bijiangensis-2     .T. ... ..A ... ..C ... ... ..T T.. ... ... ..C ... ... ..T ... ..C ... ... ...
M.b.caobangis          .T. ... ..A ... ..C ... ... ..T T.. ... ... ..C ... ... ..T ... ..C ... ... ...
A.alces                CT. ... ..A ..C ... T.. ..A ... ..G ... ... ..C ..A ... ... ..C ..C ... ..T ..T
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FIG. 1—*Continued*

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                                                                                                                840
M.moschiferus          CCA TTA AAT ACA CCT CCA CAT ATT AAA CCC GAA TGG TAC TTT CTA TTT GCA TAT GCC ATT
M.chrysogaster         ... ... ... ..G ..C ... ... ... ... ... ... ..A ... ..C ... ... ... ... ... ..C
M.fuscus               ... ... ... ..G ..C ... ... ... ... ... ... ..A ..T ..C ... ... ... ... ... ...
M.leucogaster          ... ... ... ... ..C ... ... ... ... ... ... ..A ..T ..C ... ... ... ... ... ...
M.b.bijiangensis-1     ... ... ..C ... ..A ... ... ... ... ... ... ..A ... ..C ... ... ... ... ... ...
M.b.bijiangensis-2     ... ... ..C ... ..A ... ... ... ... ... ... ..A ... ..C ... ... ... ... ... ...
M.b.caobangis          ... ... ..C ... ..A ... ... ... ... ... ... ..A ... ..C ... ... ... ... ... ...
A.alces                ... C.C ..C ... ..C ..T ... ... ..G ..T ... ... ..T ..C T.. ... ... ..C ..A ...
                                                                                                                900
M.moschiferus          CTA CGA TCA ATT CCT AAT AAA CTA GGA GGA GTA CTA GCC CTA GTT TTA TCC ATC CTA ATT
M.chrysogaster         ... ... ... ... ..C ..C ... ... ... ... ... ... ... T.. ..C ... ..T ... ... ...
M.fuscus               ... ... ... ... ..C ..C ... ... ... ... ... ... ... T.G ..C ... ... ... ... ...
M.leucogaster          ... ... ... ... ..C ..C ... ... ... ... ... ... ... T.. ..C ... ... ... ... ...
M.b.bijiangensis-1     ... ... ... ... ..C ..C ... ... ... ... ... ... ... T.. ... ... ..T ... ... ..C
M.b.bijiangensis-2     ... ... ... ... ..C ..C ... ... ... ... ... ... ... T.. ... ... ..T ... ... ..C
M.b.caobangis          ... ... ... ... ..C ..C ... ... ... ... ... ... ... T.. ... ... ..T ... ... ..C
A.alces                ... ... ... ..C ..C ... ... ... ..G ... ..C ... ... ... ... .C. ..T ... T.. ..C
                                                                                                                960
M.moschiferus          TTA ATC TTC ATA CCC CTA CTT CAC ACA TCC AAA CAA CGA AGT ATA ATA TTC CGA CCC CTT
M.chrysogaster         ... ... ..T ... ... ... ... ..T ..G ... ... ... ... ... ... ... ... ... ..T ..C
M.fuscus               ... ... ... ... ... ... ... ..T ..G ..T ... ... ... ... ... ... ... ... ..T ..C
M.leucogaster          ... ... ..T ... ... ... ... ..T ..G ..T ... ... ... ... ... ... ... ... ..T ..C
M.b.bijiangensis-1     ... ... ..T ... ... ... ... ..T ... ..T ... ... ... ... ... ... ... ... ..T ...
M.b.bijiangensis-2     ... ... ..T ... ... ... ... ..T ... ..T ... ... ... ... ... ... ... ... ..T ...
M.b.caobangis          ... ... ..T ... ... ... ... ..T ... ..T ... ... ... ... ... ... ... ... ..T ...
A.alces                C.. ..T C.. ... ..A ..C ..C ... ... ... ... ... ..C ..C ..G ... ... ... ..A T.C
                                                                                                               1020
M.moschiferus          AGC CAA TGC CTA TTC TGA ATT TTA GTA GCA GAT TTA TTG ACA CTT ACA TGA ATT GGA GGA
M.chrysogaster         ..T ... ... ... ..T ... ... ... ... ... ..C C.. C.A ... ..C ..G ... ... ... ...
M.fuscus               ... ... ... ... ..T ... ..C ... ... ... ..C C.. C.A ... ..C ... ... ... ... ...
M.leucogaster          ... ... ... ... ..T ... ..C ... ... ... ..C C.G C.A ... ..C ... ... ... ... ...
M.b.bijiangensis-1     ... ... ..T ... ..T ... ... ... ... ... ... ... C.A ... ..C ... ... ... ... ...
M.b.bijiangensis-2     ... ... ..T ... ..T ... ... ... ... ... ... ... C.A ... ..C ... ... ... ... ...
M.b.caobangis          ... ... ..T ... ..T ... ... ... ... ... ..C ... C.A ... ..C ... ... ... ... ...
A.alces                ..T ... ... ..G ... ... ... C.. ... ... ..C C.. ..A ... ..C ... ... ..C ... ...
                                                                                                               1080
M.moschiferus          CAA CCA GTT GAA CAC CCA TAT ATC ATT ATC GGA CAA CTA GCA TCC ATT ATA TAC TTT CTT
M.chrysogaster         ... ... ..C ... ... ... ... ..T ... ..T ... ... ... ... ..T ..C ... ... ... ...
M.fuscus               ... ... ..C ... ... ... ... ..T ... ..T ... ... ... ..G ..T ..C ... ... ..C ..C
M.leucogaster          ... ... ..C ... ... ... ... ..T ... ..T ... ... ... ... ..T ..C ... ... ..C ...
M.b.bijiangensis-1     ... ... ..C ... ... ... ... ..T ... ..T ... ... ... ... ..T ..C .C. ..T ... ...
M.b.bijiangensis-2     ... ... ..C ... ... ... ... ..T ... ..T ... ... ... ... ..T ..C .C. ..T ... ...
M.b.caobangis          ... ... ..C ... ... ... ... ..T ... ..T ... ... ... ... ..T ..C .C. ... ... ...
A.alces                ..G ... ... ... T.T ..T .T. ..T .CC ..T ... ... ... ... ... ..C T.. ..T ..C ..C
                                                                                                               1140
M.moschiferus          CTT ATC CTA GTA ATA ATA CCG GTA GCC AGC ATA GTC GAA AAC AAT CTC TTA AAA TGA AGA
M.chrysogaster         ... ..T ... ... ... ... ..A ... ... ... ... ..T ... ..T ... ... ... ... ... ...
M.fuscus               ... ..T ... ... ... ... ..A ... ..T ... ... ..T ... ..T ... ... ... ... ... ...
M.leucogaster          ... ..T ... ... ... ... ..A ... ... ... ... A.T ... ..T ... ... ... ... ... ...
M.b.bijiangensis-1     ... ... ... ... ... ... ... ... ... ... ... ..T ... ..T ..C ... ... ... ... ...
M.b.bijiangensis-2     ... ... ... ... ... ... ... ... ... ... ... ..T ... ..T ..C ... C.. ... ... ...
M.b.caobangis          ... ... ... ... ... ... ..A ... ... ... ... A.T ... ..T ..C ... ... ... ... ...
A.alces                A.. ... ..G ... C.T ..G ..A ..T A.T ... .CG A.. ... ..T ..C ... C.. ..G ... ...
```

FIG. 1—*Continued*

pecora groups, the available complete Cytb sequences of 21 other pecora species were chosen for analysis (Chikuni *et al.*, 1995; Irwin *et al.*, 1991; Anderson *et al.*, 1982; Tanaka *et al.*, 1996). They represent the other 4 families in the pecora group, including 1 species in the Giraffidae, 1 species in the Antilocapridae, 8 species in the Bovidae, and 11 species in the Cervidae. In addition, the sequences of 2 species in the Tragulidae were used for rooting.

RESULTS AND DISCUSSION

Cytochrome b Gene Sequences and Variations

Figure 1 shows the aligned Cytb sequences of musk deer and moose. In the eight samples sequenced, seven Cytb haplotypes were observed, while the sequences of the two *M. moschiferus* are identical even though their geographic origins are different (Table 1). Among the 1140 bp of Cytb sequences, all the sequences start with

TABLE 3

Base Composition at First, Second, and Third Positions of Codons in Musk Deer

Species	First				Second				Third				Bias[a]
	A	T	C	G	A	T	C	G	A	T	C	G	
M. moschiferus	30.3	23.2	24.5	21.9	20.3	42.7	23.5	13.5	44.9	20.8	31.7	2.6	0.355
M. chrysogaster	30.3	23.0	24.5	22.2	20.3	42.7	23.5	13.5	45.1	21.6	30.6	2.6	0.343
M. fuscus	30.3	23.2	24.3	22.2	20.3	42.7	23.5	13.5	44.6	21.4	30.9	3.2	0.339
M. leucogaster	30.6	23.0	24.5	21.9	20.3	42.7	23.5	13.5	45.6	20.6	31.7	2.1	0.364
M. b. bijiangensis-1	30.3	23.5	23.7	22.4	20.3	43.0	23.2	13.5	44.9	22.7	29.6	2.9	0.326
M. b. bijiangensis-2	30.3	23.2	24.0	22.4	20.3	43.0	23.2	13.5	44.9	22.7	29.6	2.9	0.326
M. b. caobangis	30.6	23.2	24.0	22.2	20.3	43.0	23.2	13.5	45.4	21.9	30.3	2.4	0.343
Mean	30.4	23.2	24.2	22.2	20.3	42.9	23.4	13.5	45.0	21.7	30.6	2.7	0.341

[a] Values at the third codons; the fomula for bias calculations follows Irwin *et al.* (1991).

initial codon 'TGA' and end in stop codon 'AGA,' coding 379 amino acids in length. No deletions or insertions were observed. The compositions of nucleotides for each sequence are listed in Table 3, which shows that the nucleotide composition biases are similar among the sequences. This fits the requirements of a good phylogenetic marker (Irwin *et al.*, 1991). Within the musk deer, a total of 125 sites are variable (10.96%), of which 13 sites are located at the first codons, 3 sites at the second, and 109 sites at the third. Interestingly, among the 16 sites with the first and second codon substitutions, 9 sites lead to 8 amino acid changes while the other 7 sites are synonymous substitutions of the first codon of leucine. The amino acid substitutions all happened among the hydrophobic amino acids (leucine, isoleucine, valine, threonine, and alanine), which are located mainly in the transmembrane domain of Cytb. The transition–transversion bias in musk deer was calculated to be 22 in average, falling within the spectrum of mammals (Brown *et al.*, 1982; Irwin *et al.*, 1991). The pairwise substitution matrix among musk deer is given in Table 4.

TABLE 4

Pairwise Comparisons of Sequence Divergence within Musk Deer

	1	2	3	4	5	6	7	8
1. *A. alces*		46	46	46	46	49	49	49
2. *M. moschiferus*	139		2	2	2	3	3	3
3. *M. chrysogaster*	138	81		0	0	3	3	3
4. *M. fuscus*	141	81	24		0	3	3	3
5. *M. leucogaster*	142	80	27	17		3	3	3
6. *M. b. bijiangensis-1*	143	80	47	47	44		0	0
7. *M. b. bijiangensis-2*	141	81	48	48	45	5		0
8. *M. b. caobangis*	138	81	42	42	37	11	12	

Note. The numbers below the diagonal represent transitional substitutions; those above the diagonal represent transversional substitutions.

Relative Rate Test and Molecular Time Scale

To determine the homogeneity of the molecular evolutionary rate of Cytb in musk deer, we employed the relative rate test given by Sarich and Wilson (1973). The results are shown in Table 5, which indicates a relatively even rate among all the species ($K_{AC}/K_{BC} = 1.0 \pm 0.06$). Using silent substitutions at the third position of codons and a transition to transversion ratio of 10:1 for divergences up to 25 Myr (million years), Irwin *et al.* (1991) suggested an estimation of a

TABLE 5

Relative Rate Test

OTU pairs	K_{AB}	K_{AC}	K_{BC}	K_{AC}/K_{BC}
M. m. vs *M. c.*	74	142	141	1.01
M. m. vs *M. f.*	75	142	143	0.99
M. m. vs *M. l.*	72	142	144	0.99
M. m. vs *M. b. b1*	72	142	145	0.98
M. m. vs *M. b. b2*	72	142	144	0.99
M. m. vs *M. b. c*	73	142	142	1.00
M. c. vs *M. f.*	23	141	143	0.99
M. c. vs *M. l.*	24	141	144	0.99
M. c. vs *M. b. b1*	42	141	145	0.97
M. c. vs *M. b. b2*	42	141	144	0.98
M. c. vs *M. b. c.*	37	141	142	0.99
M. f. vs *M. l.*	15	143	144	0.99
M. f. vs *M. b. b1*	43	143	145	0.99
M. f. vs *M. b. b2*	43	143	144	0.99
M. f. vs *M. c*	38	143	142	1.01
M. l. vs *M. b. b1*	38	144	145	0.99
M. l. vs *M. b. b2*	38	144	144	1.00
M. l. vs *M. c*	33	144	142	1.01
M. b1 vs *M. b. b2*	4	145	144	1.01
M. b1 vs *M. b. c*	9	145	142	1.02
M. b2 vs *M. b. c*	9	144	142	1.01

Note. The numbers are the synonymous substitutions at the third positions of codons, where *M. m* refers to *M. moschiferus, M. c., M. chrysogaster, M. l., M. leucogaster, M. f., M. fuscus, M. b. b1, M. b. bijiangensis-1, M. b. b2, M. b. bijiangensis-2,* and *M. b. c, M. b. caobangis.*

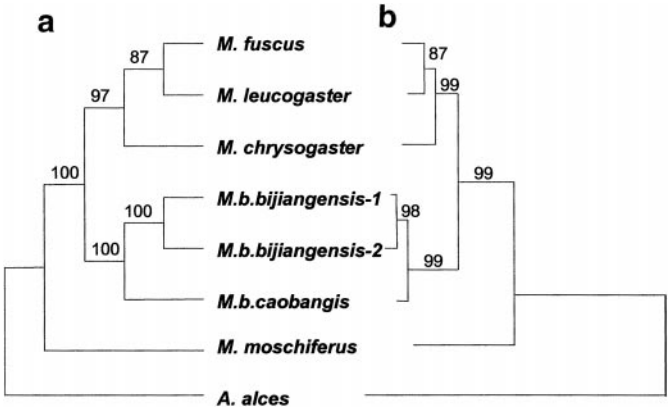

FIG. 2. Phylogenetic relationships of five species and one subspecies of musk deer. (a) The most parsimonious tree constructed from PAUP 3.0 (tree length = 297, CI = 0.862). The bootstrap values with 1000 replicates are shown above the branches. (b) The NJ tree under Kimura's 2-parameter model. The branch lengths are proportional to the genetic distances in Table 4. Numbers above internal branches are branch length confidence levels.

silent divergence rate of approximately 10% per million years in mammals. This rate was used as a molecular clock in dating the divergence events in musk deer.

Phylogenetic Analysis

In the parsimony analysis, within the seven sequences of musk deer, 85 sites (7.46%) were found to be informative. The strength of phylogenetic signals was evaluated through exhaustive search in PAUP3.0. The tree length distribution is quite structured, implying strong phylogenetic signals in the data (Hillis and Huelsenbeck, 1992). The most parsimonious tree is shown in Fig. 2a, and there are no trees, which are one or two steps less parsimonious. The topology of the most parsimonious tree is quite robust, given that the bootstrap values for internal lineage are all above 95%, except for the one clustering *M. fuscus* and *M. leucogaster* (87%). However, when we weighted transversions and transitions differently according to the average ratio in musk deer, we still had the same topology shown in Fig. 2a, but with lower bootstrap values (data not shown). We also used the maximum likelihood and neighbor-joining approaches for tree constructing. The genetic distance matrix under Kimura's 2-parameter model is listed in Table 6. Figure 2b shows the neighbor-joining tree with the confidence level of branch lengths (CP). The NJ tree is identical to the parsimonious tree in topology, and so is the maximum likelihood tree. Deer species commonly considered to be closely related to musk deer were tried for tree rooting. As a result, the topology remained unchanged for all the outgroups used while the bootstrap confidence values varied among them.

As mentioned above, morphologists have been debating the taxonomy of musk deer for decades. The limited information resulting from the morphological similarity among musk deer is one of the critical reasons for the long-standing controversy. Some morphologists identify only one species, *M. moschiferus* (Ellerman *et al.*, 1950), while others (Gao, 1963) suggest three species. Sheng (1989) proposed that *M. moschiferus* and *M. berezovskii* should be one species due to very similar skull structure. Based on distinct morphological characters, Groves (1986, 1995) suggested a five-species array for musk deer and distinguished *M. moschiferus* from the other musk deer, indicating it as a sister taxon to the other species. Generally, the molecular tree is consistent with the classification suggested by Groves *et al.* (1995), in which all five suggested species show a

TABLE 7

Matrix of Transversional Substitutions at the Third Positions of Codon among the Five Pecora Families and Tragulidae

	1	2	3	4	5	6
1. Tragulidae	—					
2. Bovidae	64.9	—				
3. Antilocapridae	68.0	45.8	—			
4. Giraffidae	69.0	36.5	37.0	—		
5. Moschidae	68.0	36.3	43.6	35.0	—	
6. Cervidae	68.3	40.5	45.1	35.2	36.0	—

TABLE 6

Matrix of Genetic Distances under Kimura's 2-Parameter Model

	1	2	3	4	5	6	7	8
1. *A. alces*		0.0149	0.0148	0.0150	0.0151	0.0153	0.0151	0.0149
2. *M. moschiferus*	0.1882		0.0090	0.0090	0.0089	0.0090	0.0091	0.0091
3. *M. chrysogaster*	0.1870	0.0788		0.0045	0.0047	0.0066	0.0067	0.0062
4. *M. fuscus*	0.1907	0.0788	0.0216		0.0037	0.0066	0.0067	0.0062
5. *M. leucogaster*	0.1919	0.0777	0.0243	0.0152		0.0064	0.0065	0.0058
6. *M. b. bijiangensis-1*	0.1964	0.0787	0.0459	0.0459	0.0430		0.0020	0.0030
7. *M. b. bijiangensis-2*	0.1940	0.0797	0.0469	0.0469	0.0440	0.0044		0.0031
8. *M. b. caobangis*	0.1903	0.0797	0.0411	0.0411	0.0364	0.0098	0.0107	

Note. The numbers below the diagonal are pairwise genetic distances, those above the diagonal are standard errors.

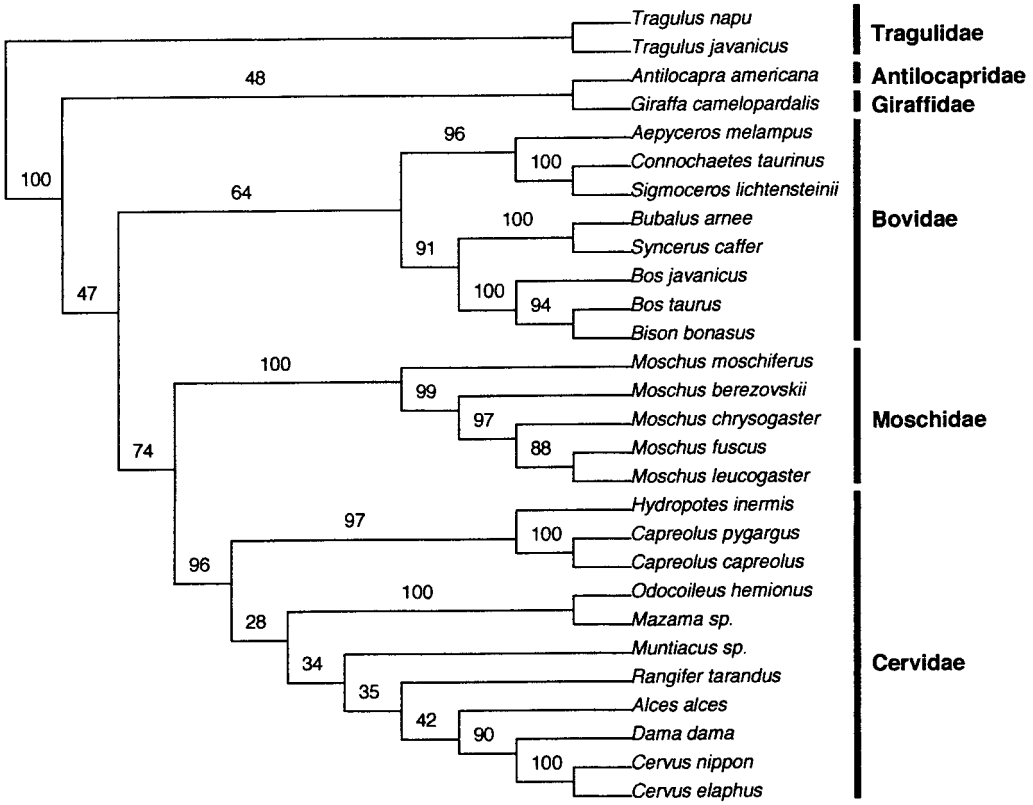

FIG. 3. The neighbor-joining tree of five pecora families, showing a close relationship between Moschidae and Cervidae. The numbers above the lineages are bootstrap values with 1000 replicates.

certain degree of genetic divergence and *M. moschiferus* is clearly distinguished from the others.

Even though the five-species suggestion was well reflected by the molecular tree, the relatively short genetic divergence among some of the species raises another issue: how much genetic divergence is expected among species? According to the RFLP study of mitochondrial DNA on deer by Cronin (1991), the mtDNA sequence divergences within species are <3% and 4–12% between species within subfamilies. Sequence data of Cytb (645 bp) from Chikuni *et al.* (1995) showed about 7% sequence divergence among three serow species (genus *Capricornis*). In our data (see Table 5), the sequence divergence between *M. moschiferus* and other species was marked at about 7% while the divergence of *M. berezovskii* from the other three species was 4%. However, the divergences among *M. chrysogaster, M. fuscus,* and *M. leucogaster* were quite low (<3%), given that they are listed as separate species by morphologists. If we accept the species status of these taxa, it appears that they were rather recent speciation events in musk deer. Interestingly, the three species all live in the areas of the Himalaya and Hengduan mountains (altitude 2800 to 4800 m), which have a rather rich biodiversity due to diverse ecological habitats caused by orogenic movements since the Pliocene. When we look at the fossil record of musk deer, the oldest specimen is from *M. moschiferus,* which was found in northern Asia and dated to be around 700,000 years old (Dong, 1993). Hence, the fossil record and the molecular dating are quite consistent with each other. By comparing the fossil evidence with our molecular data, we suggest that the historical dispersion of musk deer in China might be from north to south.

However, it should be mentioned that with closely related species, as is the case in musk deer, the mitochondrial phylogeny might partially reflect the phylogenetic relations. Interspecies introgression could also contribute to the similar morphological phenotypes while the mitochondrial genotypes remain different (Bradley *et al.*, 1996). Therefore, it will be interesting to sequence some nuclear genes in future studies.

Phylogenetic Relationships of Musk Deer with Other Pecora Groups

There have been different opinions on the taxonomic status of musk deer. Some authors suggested its genus status in Cervidae while others prefer its placement in a separate family, the Moschidae (Nowak, 1991; Corbet and Hill, 1992). We calculated the substitutions of third-codon transversions among musk deer and other pecora groups. These transversions have been shown to accumulate almost linearly with time in mammals (Irwin *et al.*, 1991). Musk deer showed levels of se-

quence divergence similar to those among other pecora families (Table 7), supporting a separate family status. Figure 3 shows the neighbor-joining tree indicating relations among the five families of pecora. The genetic distances were calculated under Kimura's 2-parameter model (data not shown). In the NJ tree, musk deer is strongly supported as a monophyletic group and is closely related to Cervidae. This result was also supported in our parsimony and maximum likelihood analyses (trees not shown). Using the divergence rate of 0.5% per Myr of transversions at third codon (Irwin *et al.*, 1991), the divergence times among the pecora groups in Fig. 3 were calculated to be from 18.4 to 24.1 Myr, reflecting the radiation event that occurred 20–25 Myr ago (Irwin *et al.*, 1991).

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