

Diversity of Mitochondrial DNA cytochrome b Gene in Two Subspecies of Striped Field Mouse, *Apodemus agrarius coreae* Thomas and *A. agrarius manchuricus* Thomas (Mammalia, Rodentia) from Korea and Northeast China

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ABSTRACT

The partial sequences of mtDNA cytochrome b gene in two subspecies of striped field mouse (*Apodemus agrarius coreae* and *A. agrarius manchuricus*) from Korea and northeast China were analyzed to determine the degree of genetic diversity in each subspecies and to confirm their subspecific difference. In 18 specimens of *A. agrarius coreae*, ten haplotypes were resulted, and in two specimens of *A. agrarius manchuricus*, one haplotype was revealed. Tamura-Nei nucleotide distance among ten haplotypes in subspecies *coreae* ranged 0.36 to 1.86%. and nucleotide distance between two subspecies (*coreae* and *manchuricus*) was 0.37 to 1.47%: maximum infrasubspecific divergence in *coreae* was greater than maximum intersubspecific difference between two subspecies. Moreover, no major subgroup was resulted when 11 haplotypes in two subspecies were compared. Our sequence result was not concordant with the morphological data studied so far, and it is concluded that cytochrome b gene sequence is not a good genetic marker to distinguish two subspecies of *A. agrarius*. In future, mtDNA control region analyses seemed to be necessary to reveal genetic relationship between these two subspecies of *A. agrarius*.

Key words: systematics, mtDNA cytochrome b gene, striped field mouse, *Apodemus agrarius*

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INTRODUCTION

The genus *Apodemus*, composed of 21 species, is confined to the Palaearctic region, and striped field mouse, *Apodemus agrarius* Pallas 1771, inhabit from Germany through China to Korea (Musser and Calton, 1993). In Korea, four subspecies of *A. agrarius* were recognized (Jones and Johnson, 1965): *manchuricus* in the extreme northern part of the peninsular, *pallidior* in the coastal lowlands of southern Korea, *coreae* throughout the major portion of the peninsula, and *chejuensis* in Cheju Island. In China, five subspecies of *Apodemus agrarius* (subspecies *agrarius*, *ningpoensis*, *pallidior*, *manchuricus*, and *insulaemus*) were reported, and subspecies *manchuricus* inhabit in northeast China and east Inner Mongolia (Xia, 1985). However, most subspecies of *A. agrarius* were designated by the differences in pelage colour or body size (Corbet, 1978), and subspecies classification of *A. agrarius* is still in confusion (Kobayashi, 1985).

Mitochondrial DNA is a highly sensitive genetic marker suitable for studies of closely related taxa or populations of a variety of species (Sunnucks, 2000). The analysis of Restriction Fragment Length Polymorphisms (RFLPs) is a powerful and cost-effective method where large numbers of individuals or segments are being screened (Dowling *et al.*, 1996). And DNA sequences have become the most frequently used taxonomic characters for inferring phylogenetic history as they are the basic units of information encoded by organisms (Hillis *et al.*, 1996). Koh *et al.* (1999) reported by mtDNA RFLP analysis with six subspecies of *A. agrarius* that *chejuensis* and *coreae* are distinct subspecies, and that subspecies *manchuricus* and *pallidior* are synonyms of subspecies *ningpoensis*.

In this study, partial sequences of mtDNA cytochrome b gene in two subspecies of striped field mouse (*Apodemus agrarius coreae* and *A. agrarius manchuricus*) from Korea and northeast China were analyzed to determine the degree of genetic diversity in each subspecies and to confirm their subspecific difference.

MATERIALS AND METHODS

Materials

Eighteen specimens of striped field mouse (*Apodemus agrarius coreae*) from seven localities in Korea (Cheongju, 3; Mt. Weolak, 2; Mt. Sobaek, 3; Mt. Chiak, 4; Mt. Odae, 2; Mt. Taebaek, 2; Mt. Seolak, 2) and two specimens of striped field mouse (*A. agrarius manchuricus*) from Changchun, northeast China were used. Muscle tissues were preserved in a deep freezer.

DNA isolation, amplification, and sequencing

From muscle samples, total cellular DNA was extracted (Hillis *et al.*, 1996): 500 μ l of STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), 25 μ l of 10 mg/ml stock of roteinase K, and 25 μ l of 20% SDS were added into a micro tube containing minced tissue. It was incubated at 55 $^{\circ}$ C for 2h, and DNA was extracted with equal volume of PCI and chloroform, and then was precipitated with 2 vols. ethyl alcohol. After adding RNase A (10 mg/ml), the solution was

incubated at 37°C for 2h, and DNA was extracted again.

The cytochrome b gene was PCR-amplified using primers L14724 and H15149, designed by Irwin *et al.* (1991). PCR thermal cycle was as follows: 94°C for 5 min; 94°C for 1 min, 57°C for 1 min, 72°C for 1 min (32 cycles); 72°C for 5 min. To remove primer and unincorporated nucleotides, amplified product was purified using DNA PrepMate™ kit with silica-based matrix (Bioneer Co., Korea).

For sequencing, the purified PCR products were analyzed with an automated DNA sequencer (Perkin Elmer 377) at Korea Basic Science Institute (Daejeon, Korea).

Data analysis

The partial sequences of mitochondrial DNA cytochrome b gene in 20 specimens of striped field mouse were resulted. Tamura-Nei distances (Tamura and Nei, 1993) were calculated and phylogenetic tree was constructed by UPGMA and neighbor-joining method with 500 bootstrapped replications using MEGA (version 1.01).

RESULTS

All 278 bp of partial sequences of mitochondrial DNA cytochrome b gene were obtained, and 11 haplotypes of two subspecies of striped field mouse (*Apodemus agrarius coreae* and *A. agrarius manchuricus*) are shown in Fig. 1. Roman numerals indicate haplotypes: haplotypes I to X is from 18 specimens of *A. agrarius coreae* in Korea, and haplotype XI is from two specimens of *A. agrarius manchuricus* from northeast China (I, 2 from Cheongju, 3 from Mt. Sobaek, 1 from Mt. Chiak, and 1 from Mt. Odae; II, 1 from Mt. Chiak; III, 1 from Mt. Weolak and 1 from Mt. Chiak; IV, 1 from Mt. Odae; V, 1 from Mt. Chiak; VI, 2 from Mt. Seolak; VII, 1 from Mt. Weolak; VIII, 1 from Mt. Taebaek; IX, 1 from Mt. Taebaek; X, 1 from Cheongju; XI, 2 from Changchun).

Table 1. Kimura-Nei distances among 11 haplotypes in two subspecies of striped field mouse (*Apodemus agrarius coreae* and *A. agrarius manchuricus*). The distances were calculated from the sequences of mtDNA cytochrome b genes. Haplotypes are labeled as in Fig. 1.

Haplotype	I	II	III	IV	V	VI	VII	VIII	IX	X
II	0.0073									
III	0.0037	0.0110								
IV	0.0073	0.0146	0.0110							
V	0.0073	0.0147	0.0036	0.0073						
VI	0.0073	0.0147	0.0110	0.0073	0.0074					
VII	0.0074	0.0147	0.0110	0.0148	0.0147	0.0147				
VIII	0.0110	0.0184	0.0147	0.0036	0.0110	0.0110	0.0186			
IX	0.0036	0.0110	0.0073	0.0036	0.0037	0.0037	0.0110	0.0074		
X	0.0036	0.0110	0.0073	0.0110	0.0110	0.0110	0.0111	0.0148	0.0073	
XI	0.0073	0.0147	0.0110	0.0073	0.0074	0.0074	0.0147	0.0110	0.0037	0.0110

I	CTATGCCTCGTAATTCAAATTCTTACAGGCTTATTCCTAGCCATACACTACACATCAGACACAATAACAG
IIA.....
III
IV
V
VI
VII
VIIIT.....
IX
X
XIG.....
I	CATTCTCATCAGTTACACATATTTGCCGAGACGTAACCTATGGATGACTAATTCGATATATACACGCTAA
II
IIIG.....
IV
VG.....
VI
VII
VIII
IX
X
XI
I	TGGAGCTTCAATATTTTTTATCTGCTTATTTCTCCATGTAGGACGAGGAATGTACTACGGATCCTATGCA
IIG.....
III
IV
V
VIA.....
VIIC.....A.....T.....
VIII
IX
X
XI
I	TTTATAGAAACATGAAATATCGGAGTAGTCCTATTATTTGCAGTCATAGCTACAGCATTATAGGCTA
II
III
IV	..C.....A.....
VA.....
VIA.....
VII
VIII	..C.....A.....
IXA.....
X	..C.....
XIA.....

Fig. 1. Sequences of mitochondrial DNA cytochrome b gene among 11 haplotypes in two subspecies of striped field mouse (*Apodemus agrarius coreae* and *A. agrarius manchuricus*). All 278 bp of partial sequences were compared. Roman numerals indicate haplotypes: haplotypes I to X is from 18 specimens of *A. agrarius coreae* in Korea and haplotype XI is from two specimens of *A. agrarius manchuricus* in northeast China. Haplotype I has been used as the reference, and dots represent identity to the reference.

As given in Table 1, Tamura-Nei nucleotide distance among ten haplotypes (I to X) of *A. agrarius coreae* ranged 0.36 to 1.86%, and nucleotide distance between *A. agrarius coreae* (ten haplotypes of I to X) and *A. agrarius manchuricus* (haplotype XI) was 0.37% to 1.47%. It was

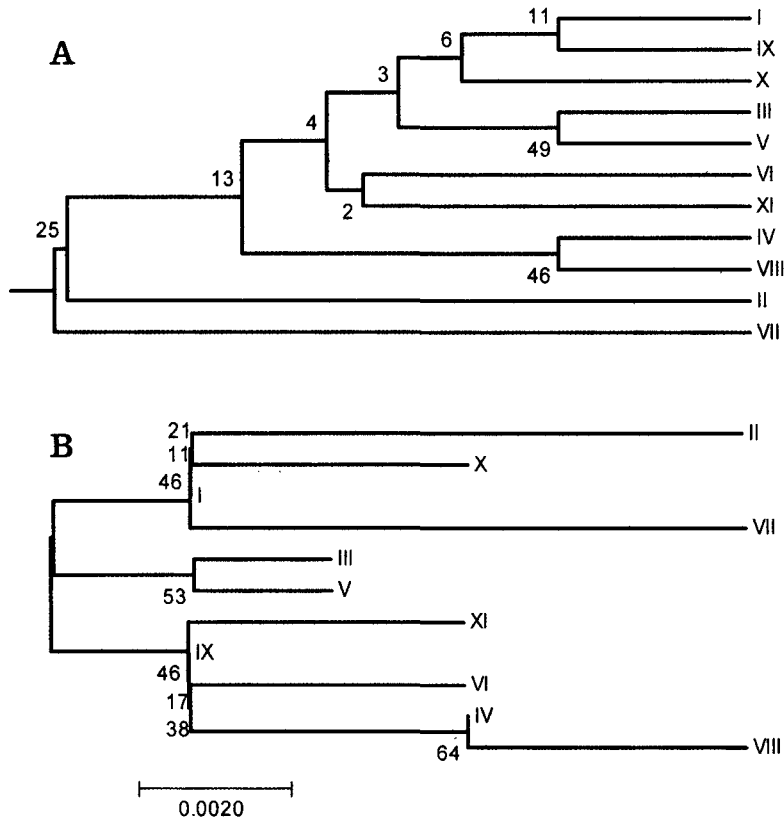


Fig. 2. The phylogenetic trees with 11 haplotypes in striped field mouse (*Apodemus agrarius coreae* and *A. agrarius manchuricus*) by UPGMA (A) and neighbor-joining method (B) with 500 bootstrap replications. Kimura-Nei distances in Table 1, calculated from mtDNA cytochrome b gene sequences in Fig. 1, were used. Haplotypes are labeled as in Fig. 1. Bootstrap values are indicated above branches, and bars indicate substitutions per site.

revealed that maximum infrasubspecific divergence in subspecies *coreae* was greater than maximum intersubspecific difference between two subspecies (*coreae* and *manchuricus*).

Eleven haplotypes in two subspecies of *A. agrarius* were compared by UPGMA (Fig. 2A) and neighbor-joining method (Fig. 2B): no distinct subgroup was resulted.

DISCUSSION

Mitochondrial DNA RFLP and cytochrome b gene sequence are useful tool for the analyses of genetic diversity within population (Hillis *et al.*, 1996). Irwin *et al.*'s (1991) analysis of mammalian mitochondrial cytochrome b gene sequences suggested that the rate of base substitution was similar to the previous estimation of 2.1% per Myr by RFLP studies (Brown *et al.*, 1979), indicating that the results based on each method are comparable with each other.

In intraspecific variation by RFLP analyses, sequence divergence of mtDNA estimated in *Rattus norvegicus* ranged from 0.2 to 0.8% (Brown and Simpson, 1981), and maximum divergence in *Clethrionomys rutilus* was 0.89% (Tegelstrom *et al.*, 1988). On the other hand, sequence divergence between *Peromyscus maniculatus* mtDNA haplotypes was as high as 7% (Lansmann *et al.*, 1983). The great intraspecific sequence divergence among mtDNA haplotypes of *A. sylvaticus* was 1.4% and that of *A. flavicolis* was 1.0% (Tegelstrom and Jaarola, 1989). Maximum mtDNA variation among six subspecies of *A. agrarius* was 1.9% (Koh *et al.*, 1999).

Maximum nucleotide divergence of mtDNA control region sequence among all samples of *A. agrarius coreae* and *A. agrarius chejuensis* was 3.5% (Koh *et al.*, 2000a). In this study with mtDNA cytochrome b gene (see Table 1), Tamura-Nei nucleotide distance among 11 haplotypes in *A. agrarius coreae* and *A. agrarius manchuricus* ranged 0.36 to 1.86%, indicating that the genetic diversity between two subspecies of *A. agrarius* is moderate.

In infrasubspecific variation by RFLP analyses, sequence variation in *Mus musculus bactrinus* was 4%, whereas *M. musculus castaneus* was 0.3% (Yonekawa *et al.*, 1981). Intrasubspecific divergence within *Apodemus agrarius chejuensis* was 0.7% (Koh and Yoo, 1992), and that within *A. agrarius coreae* was 1.5% (Koh *et al.*, 1999). In *A. peninsulae peninsulae*, divergence ranged from 0.42 to 2.01% (Koh *et al.*, 1995).

In mtDNA control region sequences, maximum nucleotide distance in *A. agrarius coreae* and *A. agrarius chejuensis* were 2.98% and 1.86%, respectively (Koh *et al.*, 2000a). In this study with mtDNA cytochrome b gene (see Table 1), maximum Tamura-Nei nucleotide distance among ten haplotypes in *A. agrarius coreae* was 1.86%, indicating that the genetic diversity within *A. agrarius coreae* is moderate.

Corbet (1978) summarized 24 nominal subspecies of *A. agrarius* into three subspecies (*agraius*, *ningpoensis* and *chevieri*). Wang (1985) concluded that subspecies *chevieri* is a distinct species, and Koh and Tikhonova (1998) suggested by morphometric analysis that 15 subspecies of *A. agrarius* can be classified into five subspecies (*agraius*, *volgensis*, *ningpoensis*, *coreae* and *chejuensis*). A subspecies is an aggregate of phenetically similar populations of a species differing taxonomically from other populations of that species (Mayr and Ashlock, 1991). And it was advocated that a classification should be the product of all available characters distributed as widely and evenly as possible over the organisms studied (Huelsenbeck *et al.*, 1996).

Mitochondrial DNA is a valuable genetic marker for studies of evolutionary relationships and the population genetic structures of species in rodents (Oshida *et al.*, 2000; Mantooth *et al.*, 2000). However, mtDNA cleavage patterns does not provide support for all of the subspecies that have been described in *Mus musculus* (Yonekawa *et al.*, 1981). The mitochondrial control region has been attractive to evolutionary biologists because of its fastest change within the animal mitochondrial genome. The subspecific difference between *A. agrarius coreae* and *A. agrarius chejuensis* was confirmed by the comparison of mtDNA control region sequences (Koh *et al.*, 2000a).

Mitochondrial cytochrome b gene is a relatively conserved gene, but differences in evolutionary rate of cytochrome b gene among species of rodents are reported (Spradling *et al.*, 2001). In spite of shortcomings of mtDNA cytochrome b gene for studying population genetics, many studies demonstrate that it can provide valuable information on the population structure of many mammals

(Wu *et al.*, 2000). In *Capreolus pygargus*, nucleotide distance between cytochrome b sequences of subspecies *tianschanicus* and those of subspecies *pygragus* was average 1.3% (Koh *et al.*, 2000b).

In this study with mtDNA cytochrome b gene (see Table 1 and Fig. 2), infrasubspecific divergence in subspecies *coreae* (maximum 1.86%) was greater than intersubspecific difference (maximum 1.47%) between subspecies *coreae* and subspecies *manchuricus*. No major subgroup was resulted when 11 haplotypes in two subspecies of striped field mouse (*A. agrarius coreae* and *A. agrarius manchuricus*) were compared.

Our sequence result was not concordant with the morphological data studied so far, and it is concluded that cytochrome b gene sequence is not a good genetic marker to distinguish these two subspecies of *A. agrarius*. Since there is only one phylogenetic history of mammalian taxa, incongruent results between morphology and molecules should be interpreted as challenges for future investigations (Adoutte *et al.*, 1998). In future, mtDNA control region analyses appeared to be necessary to reveal genetic relationship between these two subspecies of *A. agrarius*.

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한국과 북동 중국에 서식하는 등줄쥐 2아종, *Apodemus agrarius coreae* Thomas and *A. agrarius manchuricus* Thomas (포유강, 설치목)의 미토콘드리아 DNA cytochrome b 유전자의 다양성

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요 약

중국과 북동 중국에 서식하는 등줄쥐 2아종 (*Apodemus agrarius coreae*와 *A. agrarius manchuricus*)의 아종 내의 유전자 다양성의 정도를 파악하고 2아종간의 차이를 확인하기 위해, 미토콘드리아 DNA cytochrome b 유전자의 부분 염기서열을 분석하였다. 열 여덟마리의 *A. agrarius coreae*로부터 10 haplotype이, 그리고 2마리의 *A. agrarius manchuricus*로부터 1 haplotype이 밝혀졌다. 아종 *coreae*의 10 haplotype간의 Tamura-Nei nucleotide 거리는 0.36에서 1.86%였고, 2아종 *coreae*와 *manchuricus*간의 nucleotide 거리는 0.37에서 1.47%였다. 아종 *coreae*내 최대 genetic divergence 거리는 2아종간의 최대거리보다 크게 나타났다. 또한 2아종의 11 haplotype을 비교했을 때에, 뚜렷하게 아군으로 나뉘어지지 않았다. 본 염기서열의 분석 결과는 지금까지의 형태적 연구의 결과와 일치하지 않았으며, cytochrome b 유전자는 *A. agrarius*의 2아종을 구분하기에 좋은 유전자 marker가 아닌 것으로 판단됐다. 앞으로, *A. agrarius*의 2아종간의 유전적 관계를 밝히기 위해 미토콘드리아 DNA control region의 분석이 필요하다고 본다.