

Lack of mitochondrial DNA sequence divergence in two subspecies of the striped field mouse (*Apodemus agrarius manchuricus* from Northeastern China and nearby Far-eastern Russia and *A. a. coreae* from the Korean Peninsula)

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Abstract: To determine the degree of mitochondrial DNA (mtDNA) divergence between two subspecies of *Apodemus agrarius* (*A. a. manchuricus* from Northeastern China and nearby far-eastern Russia and *A. a. coreae* from the Korean peninsula) and to confirm the taxonomic status of the Korean subspecies, we obtained partial sequences of the mtDNA cytochrome *b* gene (1 054 bp) and the control region (860 bp) of these two subspecies. These sequences were compared to the corresponding haplotypes of these two subspecies, obtained from GenBank. It was observed that the *A. a. manchuricus* sequences were somewhat divergent and could be separated into two or three subgroups, and we propose further DNA analyses with additional specimens to confirm the subspecific status of *A. a. manchuricus*. Additionally, the sequences of *A. a. coreae*, from Korea, were similar to the two subgroups in *A. a. manchuricus* (one subgroup of two haplotypes in the cytochrome *b*; another subgroup of two haplotypes in the control region), from Longjiang and Harbin in Northeastern China, indicating that the genetic diversity of these mice based on mtDNA sequencing is not concordant with their current classification based on morphological characters. We conclude that *A. a. coreae* from Korea is an endemic subspecies, with only morphological distinctness from *A. a. manchuricus*, though we propose further analyses with other DNA markers to reexamine the subspecies status of *A. a. coreae*. We also conclude that the Korean peninsula did not act as a refugium for *A. agrarius* during the last glaciation.

Key words: *Apodemus agrarius*; Control region; Cytochrome *b* gene; DNA systematics; Korea; Northeastern China; Striped field mouse

黑线姬鼠两亚种（中国东北地区及俄罗斯远东地区的东北亚种和朝鲜半岛的朝鲜亚种）的线粒体 DNA 序列缺少趋异性

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摘要: 为了检测黑线姬鼠两亚种（来自中国东北地区、俄罗斯远东地区的东北亚种和朝鲜半岛的朝鲜亚种）线粒体 DNA 的变异水平并确定朝鲜亚种的分类地位，我们测序分析了两亚种的线粒体 DNA 细胞色素 *b* 的部分序列（1 054 bp）和控制区的部分序列（860 bp），并与基因库中黑线姬鼠相应的单倍型序列进行了比较。可以看出东北亚种的序列显示出某些分异，可以被分为 2 或 3 个亚群，所以我们提出需要更多标本的 DNA 分析来确定东北亚种的分类地位。另外，来自韩国的朝鲜亚种的序列，与来自中国东北地区龙江和哈尔滨的东北亚种的两个亚群相似（1 个亚群是细胞色素 *b* 的两个单倍型，另 1 个是控制区的两个单倍型），表明基于线粒体 DNA 序列的遗传多样性与现今基于形态特征对这些姬鼠的分类所得结果是不一致的。因此我们认为来自韩国的朝鲜亚种是一个只在形态特异上不同于东北亚种的地方亚种，我们建议通过其他 DNA 标记来进一步验证其亚种地位。我们还认为朝鲜半岛不是最近的冰川期黑线姬鼠残遗种的保护区。

关键词: DNA 系统学; 细胞色 *b* 基因; 控制区; 黑线姬鼠; 韩国; 中国东北

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1 Introduction

The geographic distribution of the striped field mouse (*Apodemus agrarius* Pallas, 1771) extends from Germany through China to Korea (Musser and Carleton, 2005), and the subspecies classification of *A. agrarius* is still unclear because most subspecies of *A. agrarius* were designated based on differences in pelage color or body size (Kobayashi, 1985). Corbet (1978) arranged 24 nominal subspecies of *A. agrarius* into three subspecies (*agrarius*, *ningpoensis*, and *chevrieri*), but Wang (1985) concluded that the subspecies *chevrieri* is a distinct species [which was also a conclusion documented by Musser *et al.* (1996), and further discussed by Musser and Carleton (2005)]. In addition, Jones and Johnson (1965) reported that *A. a. coreae*, from the Korean peninsula, is morphologically distinct from *A. a. mantchuricus*, which has a geographically adjacent distribution in Northeastern China, and Koh and Tikhonova (1998) classified 15 subspecies of *A. agrarius* into five subspecies (*agrarius*, *volgensis*, *ningpoensis*, *coreae*, and *chejuensis*) based on morphometric analyses.

DNA sequences have become the most frequently used taxonomic characters for inferring phylogenetic history because they are the basic units of information encoded by organisms (Hillis *et al.*, 1996). Mitochondrial DNA is a highly sensitive genetic marker that

is suitable for studies of closely related taxa or populations of a variety of species (Sunnucks, 2000). In particular, control region sequencing has been found to be a valuable tool for analyzing the phylogenetic relationships among very closely related species in the subgenus *Sylvaemus* of the genus *Apodemus* (Belliniva, 2004).

The genetic distinctness between *A. a. coreae* and *A. a. chejuensis* was confirmed by a comparison of mtDNA control region sequences (Koh *et al.*, 2000), but *A. a. coreae* was not different from *A. a. mantchuricus* based on 278 bp partial mtDNA cytochrome *b* sequences (Koh *et al.*, 2001). However, Serizawa *et al.* (2002) reported the genetic distinctness of *A. a. coreae* from the subspecies *mantchuricus* without the presentation of any data to support this claim, and they suggested that the Korean peninsula acted as a refugium for rodents, including *A. agrarius*, during the last glaciation.

In this study, partial sequences of the mtDNA cytochrome *b* gene and control region from the striped field mice from Korea (*A. a. coreae*) and Northeastern China (*A. a. mantchuricus*) were obtained, and these sequences were compared to the corresponding sequences of the two subspecies of *A. agrarius* from GenBank to determine the degree of their genetic divergence and to reexamine the taxonomic status of *A. a. coreae*.

Table 1 The location, specimen number, and haplotypes of the two subspecies of *Apodemus agrarius* used in this study. Thirty-nine specimens of *A. a. coreae* from Korea and ten specimens of *A. a. mantchuricus* from northeastern China were used, and the corresponding haplotypes of the cytochrome *b* gene¹ and the control region² for each specimen are also given

Location	Specimen number (haplotype)
Mt. Jiri	KJ1449 (KCRJR01 ²) and KJ1450 (KCRJR02 ²)
Mt. Songri	KS1663 (KCBSR01 ¹ , KCRSR01 ²), KS1665 (KCRSR02 ²), KS1712 (KCRSR03 ²), KS1763 (KCRSR04 ²), KS1901 (KCBSR02 ¹ , KCRSR05 ²), KS1902 (KCBSR02 ¹ , KCRSR01 ²), and KS1903 (KCBSR02 ¹ , KCRSR06 ²)
Cheongju	KC1770 (KCBCJ01 ¹ , KCRCJ01 ²), KC177 (KCBCJ01 ¹ , KCRCJ01 ²), KC1774 (KCBCJ02 ¹ , KCRCJ02 ²), KC1780 (KCBCJ02 ¹ , KCRCJ03 ²), KC1781 (KCBCJ02 ¹ , KCRCJ04 ²), and KC1803 (KCBCJ03 ¹ , KCRCJ05 ²)
Mt. Weolak	KW1418 (KCBWA01 ¹), KW1595 (KCBWA02 ¹), KW1596 (KCBWA02 ¹ , KCRWA01 ²), KW1597 (KCBWA03 ¹ , KCRWA01 ²), KW1598 (KCBWA02 ¹ , KCRWA02 ²), KW1877 (KCBWA04 ¹ , KCRWA03 ²), KW1878 (KCBWA05 ¹ , KCRWA03 ²), KW1880 (KCBWA06 ¹ , KCRWA04 ²), and KW1881 (KCBWA07 ¹ , KCRWA05 ²)
Mt. Chiak	KC1361 (KCBCA10 ¹), KC1362 (KCBCA01 ¹), KC1363 (KCBCA02 ¹), KC1364 (KCBCA03 ¹), KC1382 (KCBCA04 ¹), KC1383 (KCBCA04 ¹ , KCBCA01 ²), KC1384 (KCBCA05 ¹ , KCBCA02 ²), KC1887 (KCBCA06 ¹ , KCBCA03 ²), KC1888 (KCBCA07 ¹ , KCBCA04 ²), KC1889 (KCBCA08 ¹ , KCBCA05 ²), and KC1890 (KCBCA09 ¹ , KCBCA06 ²)
Mt. Sobaek	KS1358 (KCBSB01 ¹ , KCBSB01 ²)
Munsan	KM1589 (KCBMS01 ¹ , KCRMS01 ²), KM1590 (KCBMS01 ¹ , KCRMS02 ²), and KM1591 (KCBMS01 ¹ , KCRMS03 ²)
Lo gjia g	CL2072 (CC LJ01 ¹ , CC LJ01 ²) and CL2073 (CCB J0 ¹ , CCR J0 ²),
Harbin	CH2063 (CCBHB02 ¹ , CCRHB01 ²), CH2064 (CCBHB01 ¹), CH2065 (CCBHB02 ¹), CH2066 (CCBHB02 ¹ , CCRHB01 ²), CH2067 (CCBHB01 ¹), and CH2068 (CCBHB01 ¹)
Daqing	CD2061 (CCBDQ01 ¹ , CCRDQ01 ²) and CD2062 (CCBDQ01 ¹ , CCRDQ01 ²)

2 Materials and Methods

For mtDNA analyses, we collected 39 specimens from seven sites in Korea and 10 specimens from three sites in Northeastern China. The collection sites (1: Mt. Jiri; 2: Mt. Songri; 3: Cheongju; 4: Mt. Weolak;

5: Mt. Chiak; 6: Mt. Sobaek; 7: Munsan; 8: Longjiang; 9: Harbin; and 10: Daqing) are shown in Fig. 1, and the specimen number of each locality is listed in Table 1. Small pieces of muscle were collected and preserved in a deep freezer at -60°C .

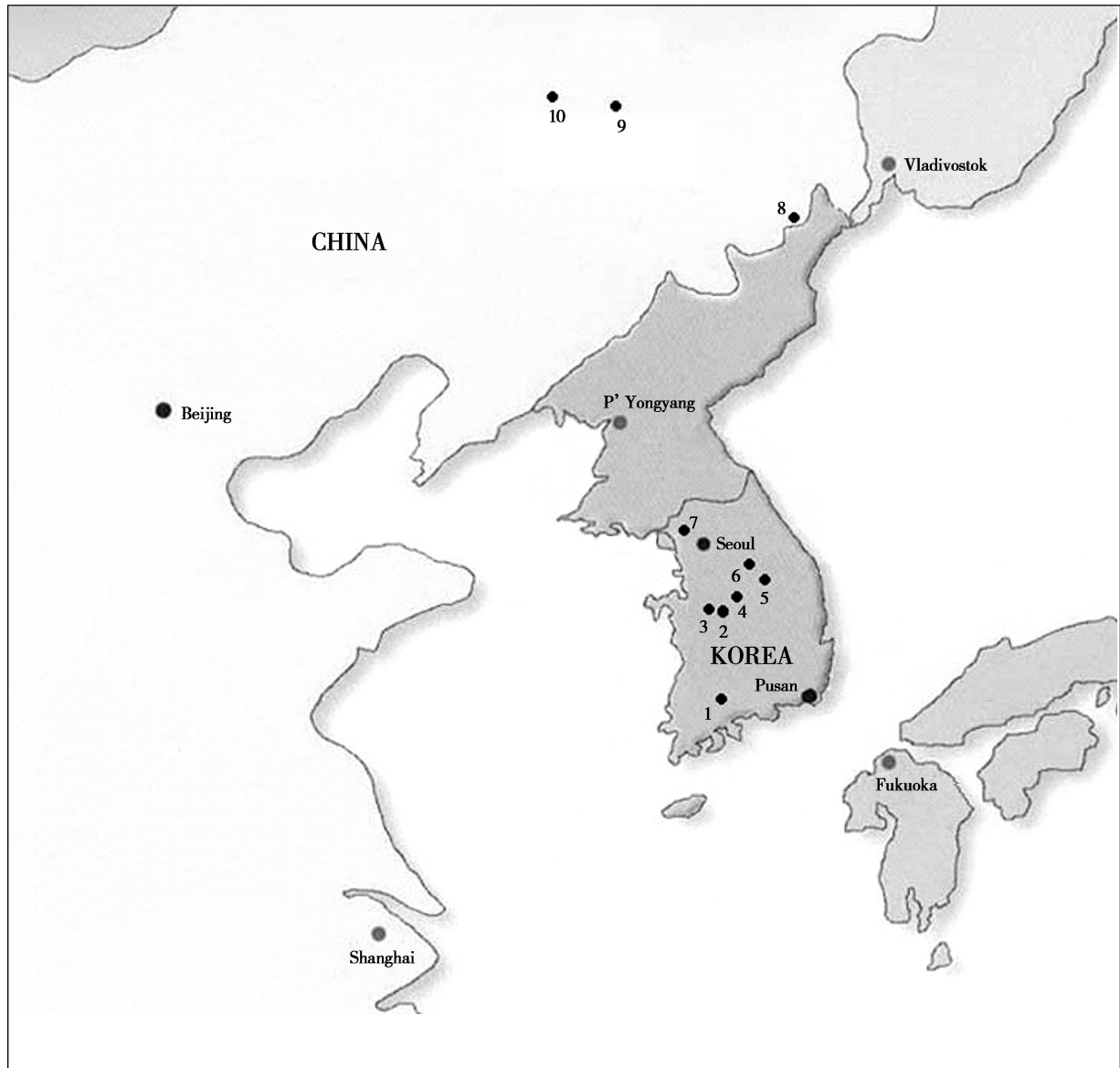


Fig. 1 Collection sites for the two subspecies of *Apodemus agrarius* used in this study. The seven sites in Korea (*A. a. coreae*) were Mt. Jiri (1), Mt. Songri (2), Cheongju (3), Mt. Weolak (4), Mt. Chiak (5), Mt. Sobaek (6), and Munsan (7), and the three sites in Northeastern China (*A. a. manchuricus*) were Longjiang (8), Harbin (9), and Daqing (10). The specimen number of each locality is listed in Table 1.

Total cellular DNA was extracted using a genomic DNA extraction kit (Intron Co., Korea). For DNA amplification, the primers L14724 and H15149 (Irwin *et al.*, 1991) were used for the cytochrome *b* gene, and the PCR thermal cycle employed 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); and 72°C for 5 min. For control region amplification, the primers Cb-Z and D4 (Shields and Kocher, 1991) were used, and the PCR thermal cycle was as follows: 94°C for 5 min;

94°C for 1 min, 59°C for 1 min, 72°C for 1 min (40 cycles); and 72°C for 5 min. To remove the primers and unincorporated nucleotides, the amplified products were purified using a DNA PrepMate™ kit with a silica-based matrix (Intron Co., Korea). For sequencing, the purified PCR products were analyzed with an automated DNA sequencer (Perkin Elmer 377) at Bioneer Co. (Seoul, Korea).

Partial sequences of the cytochrome *b* (1 054 bp) and the control region (8 600 bp) were thus obtained

from *A. a. mantchuricus* from Northeastern China and *A. a. coreae* from Korea, and these sequences were compared to the three corresponding cytochrome *b* sequences of two subspecies of *A. agrarius* obtained from GenBank [two haplotypes of *A. a. mantchuricus* (AB032851 from Ussurisky, far-eastern Russia and AF427334 from Khabarovsk, far-eastern Russia) and one haplotype of *A. a. coreae* (AB303225 from Korea) were available only for the cytochrome *b* gene].

Tamura-Nei nucleotide distances (Tamura and Nei, 1993) were calculated, and phylogenetic trees were constructed using a neighbor-joining method with 1 000 bootstrapped replications in MEGA version 3. 0 (Kumar *et al.* , 2004). *A. peninsulae* (AY389002 for cytochrome *b* and AY588251 for the control region) was used as an outgroup, and *A. a. insulaemus* (AB096816 for cytochrome *b*) and *A. a. agrarius* (AY588250 for the control region) were also used for comparisons.

3 Results

In the cytochrome *b* sequence analysis, 24 haplotypes (KCBSR01-KCBMA01) were identified from 34 specimens at six locations for *A. a. coreae*, and 5 haplotypes (CCBLJ01-CCBDQ01) were obtained from 10 specimens at three locations for *A. a. mantchuricus*, as shown in Table 1. A neighbor-joining tree with the cytochrome *b* sequences of 32 haplotypes from the two subspecies of *A. agrarius*, including three haplotypes from GenBank, is shown in Fig. 2.

The sequences from *A. a. mantchuricus* from Northeastern China and nearby far-eastern Russia were somewhat divergent and could be separated into three subgroups [mantchuricus I (haplotypes CCBLJ01 from Longjiang and CCBHB02 from Harbin); mantchuricus II (haplotypes CCBDQ01 from Daqing, CCBHB01 from Harbin, CCBLJ02 from Longjiang, and AB032851 from Ussurisky, far-eastern Russia); and mantchuricus III (haplotype AF427334 from Khabarovsk, far-eastern Russia)]. In addition, *A. a. coreae* from Korea was similar to the subgroup mantchuricus I, with an average distance of 0. 56% ; the distance between the subgroup mantchuricus I and the subgroup mantchuricus II was 0. 99% .

In the control region sequence analysis, 27 haplotypes (KCRJR01-KCRMS03) were identified from 32 specimens at seven locations for *A. a. coreae*, and 4 haplotypes (CCRLJ01-CCRDQ01) were obtained from 6 specimens at three locations for *A. a. mantchuricus*, as listed in Table 1. In a neighbor-joining tree with the control region sequences of 31 haplotypes from the two subspecies of *A. agrarius* (Fig. 3), the sequences of *A. a. mantchuricus* from Northeastern China appeared to be somewhat divergent and could be separated into two

subgroups [mantchuricus I (haplotypes CCRLJ01 from Longjiang and CCRHB01 from Harbin) and mantchuricus II (haplotypes CCRDQ01 from Daqing and CCRLJ02 from Longjiang)]. In addition, *A. a. coreae* from Korea was similar to the subgroup mantchuricus I, with an average distance of 1. 50% ; the distance between the subgroups mantchuricus I and mantchuricus II was 1. 49% .

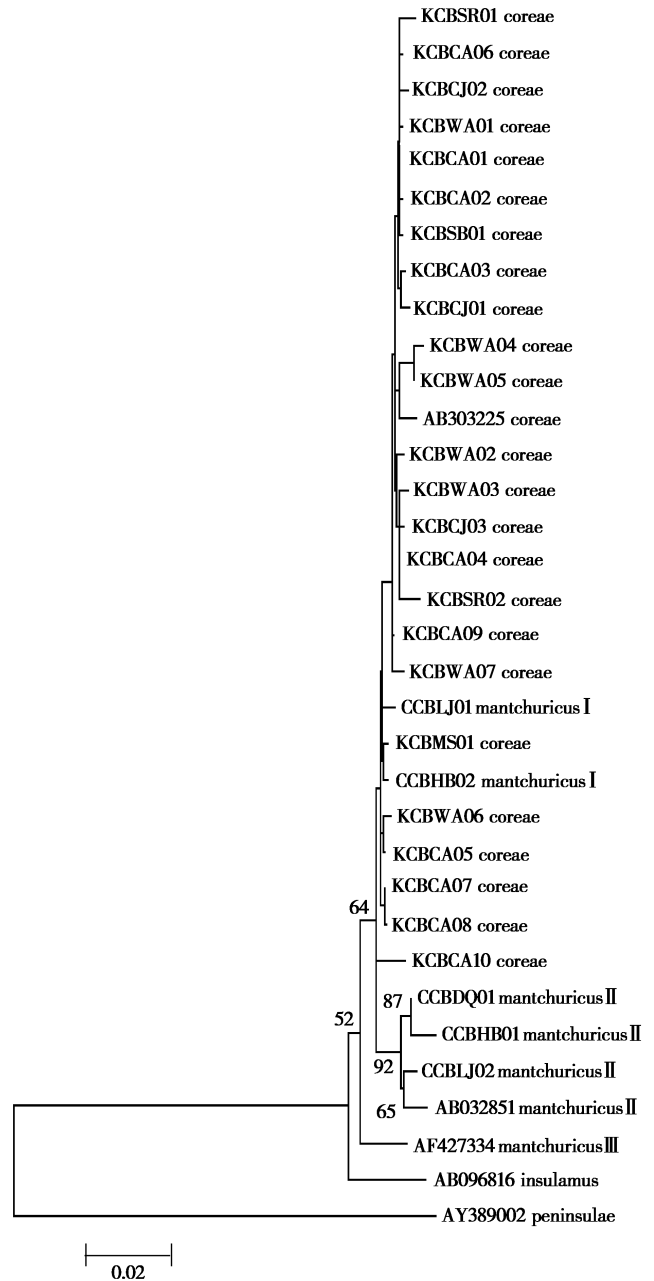


Fig. 2 Neighbor-joining tree with 32 haplotypes of the mitochondrial DNA cytochrome *b* gene in the two subspecies of *Apodemus agrarius*. Twenty-four haplotypes of *A. a. agrarius* and five haplotypes of *A. a. mantchuricus* were identified in this study, and three haplotypes of the two subspecies of *A. agrarius* were obtained from GenBank. A neighbor-joining tree with 1 000 bootstrapped replications was constructed, and the bootstrap values greater than 50% are reported at the internodes [*A. peninsulae* was used as an outgroup and *A. a. insulaemus* (AB096816) was used for comparison]. The location and specimen number for each haplotype are listed in Table 1.

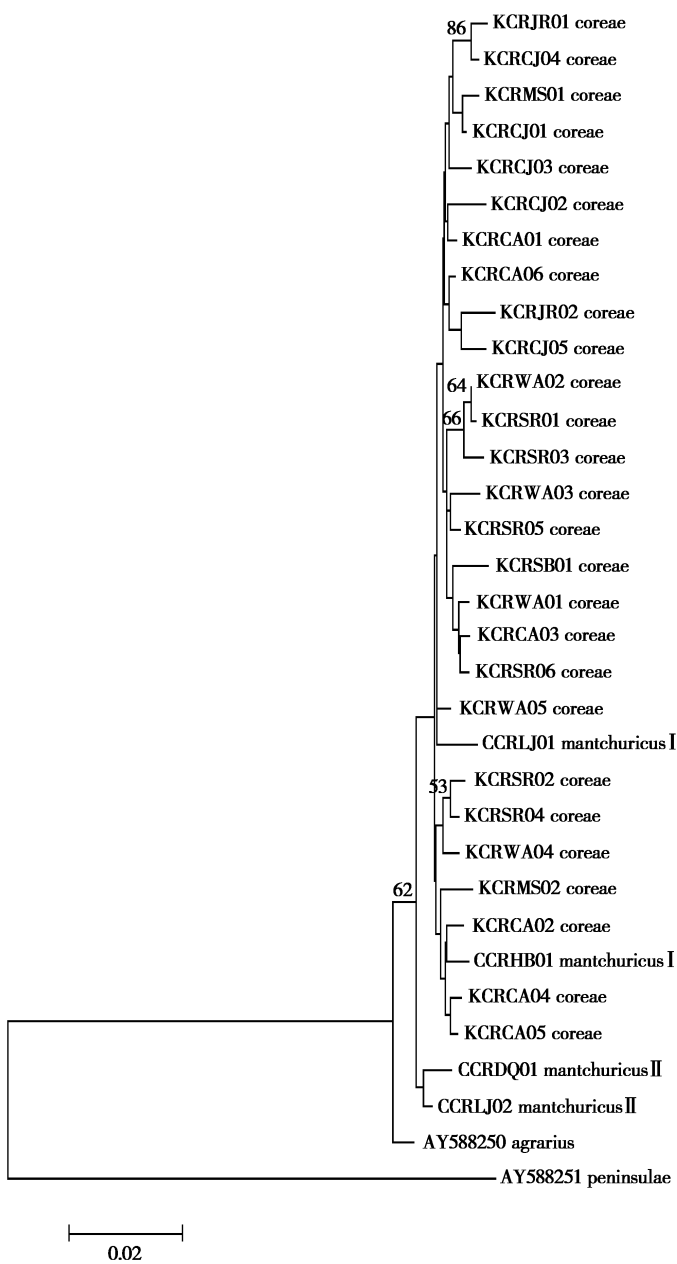


Fig. 3 Neighbor-joining tree with 31 haplotypes of the mitochondrial DNA control region in the two subspecies of *Apodemus agrarius*. Twenty-seven haplotypes of *A. a. agrarius* and four haplotypes of *A. a. mantchuricus* were identified in this study. A neighbor-joining tree with 1 000 bootstrapped replications was constructed, and bootstrap values greater than 50% are reported at the internodes [*A. peninsulae* was used as an outgroup, and *A. a. agrarius* (AY588250) was used for comparison]. The location and specimen number for each haplotype are listed in Table 1.

4 Discussion

From our analyses using 32 haplotypes of the cytochrome *b* gene and 31 haplotypes of the control region from two subspecies of *A. agrarius* (Fig. 2 and 3), we found that these two subspecies were not distinct and could not be divided into two major clades. Four haplotypes of *A. a. mantchuricus* from Longjiang and Harbin (two haplotypes in cytochrome *b* and two haplotypes in the control region) showed similarity to all of

the corresponding haplotypes of *A. a. coreae* from Korea (the average nucleotide distances between these sequences were 0.56% in the cytochrome *b* gene and 1.50% in the control region), indicating that *A. a. coreae* from Korea is not genetically distinct from *A. a. mantchuricus*. We conclude that the Korean peninsula did not act as a refugium for *A. agrarius* during the last glaciation, in contrast to what has been suggested by Serizawa *et al.* (2002).

A subspecies is an aggregate of phenetically similar populations of a species that differ taxonomically from other populations of that species (Mayr and Ashlock, 1991), and it has been 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). The shortcomings of using mtDNA sequence have been pointed out based on investigations of the systematics of hares (genus *Lepus*), and it was recommended that nuclear gene pool evidence should also be included in these analyses (Slimen *et al.*, 2008).

A. a. coreae from Korea is distinct in morphological characters from *A. a. mantchuricus*, as reported by Jones and Johnson (1965) and Koh and Tikhonova (1998), but *A. a. coreae* was previously found to not differ from *A. a. mantchuricus* with respect to partial mtDNA cytochrome *b* sequences of 278 bp (Koh *et al.*, 2001). In the present mtDNA analyses of sequences of the cytochrome *b* gene (1 054 bp) and the control region (860 bp) (Fig. 2 and 3), we confirmed that *A. a. coreae* does not differ from *A. a. mantchuricus* with respect to these sequences, which is not congruent with the current subspecies classification of these mice based on morphological characters. Thus, we conclude that *A. a. coreae* from Korea is an endemic subspecies with only morphological (phenetic) distinctness from *A. a. mantchuricus*, and we propose further analyses with other DNA markers to reexamine the subspecies status of *A. a. coreae*.

In our analyses of the cytochrome *b* and the control region sequences of *A. agrarius* (see Fig. 2 and 3), we found that the sequences of *A. a. mantchuricus* were somewhat divergent and could be separated into two or three subgroups based on these mtDNA markers. Thus, we propose further analyses with additional specimens from their distribution range to clarify the taxonomic status of these genetically divergent mice classified as *A. a. mantchuricus*.

References:

- Belliniva E. 2004. A phylogenetic study of the genus *Apodemus* by sequencing the mitochondrial DNA control region. *J Zool Syst Evol Research*, **42**: 289–297.
- Corbet G B. 1978. The mammals of the Palaearctic region: a taxonomic review. British Museum (Nat Hist). London: Cornell University

- Press, 137 – 138.
- Hillis D M, Moritz C, Mable B K. 1996. Molecular systematics, 2nd edn. Sinauer Associates, Inc., Mass. 655.
- Huelsenbeck J P, Bull J J, Cunningham C W. 1996. Combining data in phylogenetic analysis. *TREE*, **11**: 152 – 157.
- Irwin D M, Kocher T D, Wilson A C. 1991. Evolution of the cytochrome *b* gene of mammals. *J Mol Evol*, **32**: 128 – 144.
- Jones J K, Johnson D H. 1965. Synopsis of the lagomorphs and rodents of Korea. *Univ Kansas Publ Mus Nat Hist*, **16**: 357 – 407.
- Kobayashi T. 1985. Taxonomic problems in the genus *Apodemus* and its allies. In: Kawamichi T ed. Contemporary Mammalogy in China and Japan. Mamm. Soc. Japan. 80 – 83.
- Koh H S, Tikhonova G. 1998. Morphometric analyses with 15 subspecies of striped field mice, *Apodemus agrarius* Pallas (Mammalia, Rodentia) from Eurasia. *Korean J Syst Zool*, **14**: 341 – 355.
- Koh H S, Lee W J, Kocher T. 2000. Genetic relationships between two subspecies of *Apodemus agrarius* from Korea. *Heredity*, **76**: 563 – 570.
- Koh H S, Wang J, Lee B K, Heo S W. 2001. Diversity of mitochondrial cytochrome *b* gene in two subspecies of striped field mouse (*Apodemus agrarius coreae* and *A. a. mantchuricus*) from Korea and north-east China. *Korean J Syst Zool*, **17**: 49 – 57.
- Kumar K, Tamura K, Nei M. 2004. MEGA3: Integrated software for molecular evolutionary analysis and sequence alignment. *Briefings in Bioinformatics*, **5**: 150 – 163.
- Mayr E, Ashlock P D. 1991. Principles of Systematic Zoology. New York: McGraw-Hill Inc, 475.
- Musser G G, Carleton M D. 2005. Superfamily Muroidea, In: Wilson D E, Reeder D M eds. Mammal Species of the World: A Taxonomic and Geographic Reference, 3rd edn. Baltimore: Johns Hopkins University Press, vol. 2, 894 – 1531.
- Musser G G, Brothers E M, Carleton M D, Hutterer R. 1996. Taxonomy and distributional records of Oriental and European *Apodemus*, with a review of the *Apodemus-Sylvaemus* problem. *Bonner Zoologischen Beitrage*, **46**: 143 – 190.
- Serizawa K, Suzuki H, Iwasa M A, Tsuchiya K, Pavlenko M V, Kartavtseva I V, Chelomina G N, Dokuchaev N E, Han S. 2002. A spatial aspect on mitochondrial DNA genealogy in *Apodemus peninsulae* from east Asia. *Biochem Genetics*, **40**: 149 – 161.
- Shields G F, Kocher T D. 1991. Phylogenetic relationships of North American ursids based on analysis of mitochondrial DNA. *Evolution*, **45**: 218 – 221.
- Slimen H B, Suchentrunk F, Elgaaied A B A. 2008. On shortcomings of using mtDNA sequences for the systematics of hares (genus *Lepus*): an example from cape hares. *Mamm Biol*, **73**: 25 – 32.
- Sunnucks P. 2000. Efficient genetic markers for population biology. *TREE*. **15**: 199 – 203.
- Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*, **10**: 512 – 526.
- Wang Y. 1985. Subspecific classification and distribution of *Apodemus agrarius* in Sichuan, China, In: Kawamichi T ed. Contemporary Mammalogy in China and Japan. Mamm. Soc. Japan. 86 – 89.