

A Spatial Aspect on Mitochondrial DNA Genealogy in *Apodemus peninsulae* from East Asia¹

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Apodemus peninsulae is a field mouse that inhabits the broad-leaved forests of temperate Eurasia. We examined the mitochondrial cytochrome *b* gene in 57 individuals of *A. peninsulae* from northeastern Asia, including Siberia, Primorye, Magadan region, Sakhalin, Hokkaido, and the Korean Peninsula. The genealogy of the mitochondrial DNA (mtDNA) in *A. peninsulae* was shown to have substantial geographic affinity, suggesting geographic architecture of northeastern Asia, including the islands of Sakhalin and Hokkaido, played important roles on the cladogenesis. Taking into account the presence of region-specific anciently divergent mtDNA types, three parts of the regions of Primorye, Siberia, and the Korean Peninsula can be denoted as refugia for *A. peninsulae* during the substantial period of the Quaternary glacial ages. Among the geographic regions examined, Primorye is likely to be the most influential one, from which the mtDNA is thought

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to have migrated to the neighboring regions of Sakhalin, Hokkaido, the Magadan region, and Siberia during the evolution of this species.

KEY WORDS: field mice; *Apodemus peninsulae*; mitochondrial DNA; *cyt b*; molecular phylogeny; Far East Asia.

INTRODUCTION

Small mammals in northeastern Asia are interesting subjects for biogeography. The region comprises many separate geographic regions, such as Siberia, the Korean Peninsula, and Primorye in the continental part and the Sakhalin and Hokkaido islands in the peripheral part, where many mammalian species are known to exhibit substantial differentiation in morphological and genetic characteristics. The genetic analysis of species group spread across these regions would provide useful clues for understanding the role of historical and local ecological factors in structuring populations. So far we have examined three species of red-backed voles, *Clethrionomys rex*, *C. rufocanus*, and *C. rutilus*, that inhabit Hokkaido and the Russian Far East, using molecular phylogenetic markers of mitochondrial DNA (mtDNA) and nuclear ribosomal RNA genes (Iwasa *et al.*, 2000, 2002; Suzuki *et al.*, 1999; Wakana *et al.*, 1996) and have found that the geographic regions of the Korean Peninsula, Primorye, Sakhalin, and Hokkaido tend to possess region-specific genetic elements. In *C. rufocanus*, for example, substantial amounts of sequence divergences in the mitochondrial cytochrome *b* (*cyt b*) gene, as much as 2–4%, can be observed among individuals from these areas, implying that considerable time has passed since their ancestors colonized these geographic areas (Iwasa *et al.*, 2000). Hence, these geographically allocated genetic structure would be due to historical isolation in glacial refugia. To test the hypothetical view that there exist multiple Pleistocene refugia in northeastern part of Asia, we wish to know the genetic profile of another muroid rodent, the North East Asian wood mouse, *Apodemus peninsulae*, that inhabits Hokkaido as well as wide areas of the northern part of Far East Asia.

A. peninsulae inhabits the northernmost part of the range of east-Palaeartic species of *Apodemus*, and its evolutionary history might be attributed to long-term environmental changes including those of the Quaternary glacial ages. Elucidating the evolutionary histories of organisms should provide insight into ancient global environmental changes (Conroy and Cook, 2000; Hewitt, 2001; Patton *et al.*, 2000).

The species exhibits geographic variation morphologically, cytogenetically, and biochemically. *A. peninsulae* from each part of Far East Asia, including Siberia, mainland Russian Far East, the Korean Peninsula, Sakhalin, and Hokkaido are sometimes classified as different subspecies according to morphological characteristics (Vorontsov *et al.*, 1977). This species is also known to have intriguing variations in B-chromosomes (Abe *et al.*, 1997; Bekasova and Vorontsov, 1975;

Hayata, 1973; Kral, 1971; Vorontsov *et al.*, 1977). The composition of the B-chromosomes varies among individuals in the same population but tends to be specific to each population in numbers and morphology (Abe *et al.*, 1997; Bekasova *et al.*, 1980; Borisov, 1990; Hayata *et al.*, 1973; Kartavtseva *et al.*, 2000; Kartavtseva and Roslik, 1993; Volobuev, 1979). The C-band pattern of autosomes is also known to differ among populations of Sakhalin and other parts of Russia (Kartavtseva, 1999). In addition, the allelic frequency of the transferrin gene (Tf) has been revealed to differ among populations of Siberia, the Russian Far East, and Sakhalin (Pavlenko, 1989; Pavlenko and Kartavtseva, 1998).

Recently, we constructed a phylogenetic framework for the main lineages of Asian *Apodemus* species, including *A. peninsulae*, using mitochondrial and nuclear gene sequences and showed that the Asian species have long evolutionary lineages that go back to the late Tertiary in their origin (Serizawa *et al.*, 2000). Here, we examine the intraspecific variation in the *cyt b* gene of mtDNA with *A. peninsulae* samples collected from six different geographic regions: Siberia, Primorye, Magadan region, Sakhalin, Hokkaido, and the Korean Peninsula. We observed a considerable extent of intraspecific variation in the mitochondrial gene and discuss its historical episodes.

MATERIALS AND METHODS

The localities where samples of *A. peninsulae* were collected are shown in Table I and Fig. 1. We determined the nucleotide sequence for a 402-bp fragment of the *cyt b* gene in 57 individuals of *A. peninsulae*. Individuals were sampled from the six geographic regions: Siberia, Primorye, Magadan, Sakhalin, Hokkaido, and the Korean Peninsula (Table I). To further define the genetic relationships among the geographic populations with finer resolution, we sequenced the entire *cyt b* gene (1143 bp) for 25 representative individuals (Table I). Sequences for the *cyt b* gene of *A. agrarius*, *A. semotus*, and *A. speciosus* from Serizawa *et al.* (2000) were used as an outgroup in this study.

A 1.2-kb fragment of the gene for *cyt b* was first amplified with the universal primers L14724 and H15915 (Irwin *et al.*, 1991), in which the letters L and H refer to the light and heavy strands, and each number refers to the position of the 3' base of the primer in the complete sequence of human mtDNA (Anderson *et al.*, 1981). In the second PCR, nested polymerase chain reactions (PCRs) were performed according to methods previously described (Suzuki *et al.*, 1997). Single segments were amplified from the first PCR products, with the nested primer set, R-L14724 and U-H15155 (Suzuki *et al.*, 1997) with the sequence of the 18-meric dye-labeled primer, M13RP1 (R) or -21M13 (U), from ABI (a division of Perkin Elmer), which was attached to the 5' end of each of the gene-specific primers. Both strands of the product of the second PCR then were sequenced directly by

Table I. Samples Used and Types of the Mitochondrial Cytochrome *b* Gene (402 bp)

Locality	<i>n</i>	cyt <i>b</i> type
Hokkaido, Japan		
1 Shibeche	3	R16, ^a R16, ^a R16
2 Kushiro	2	R16, R16
3 Hayakita	2	R16, ^a R16
4 Tomakomai	1	R16 ^a
Sakhalin, Russia		
5 Okha	3	R14, ^a R14, R15 ^a
6 Langry river	2	R1, ^a R1
7 Sokal	1	R1 ^a
Magadan, Russia		
8 Magadan	2	R11, ^a R11
Primorye, Russia		
9 Krasnoye, Lower Amur Region	3	R5, R5, R6
10 Sovetskaya Gavan'	1	R1 ^a
11 Birakan, Middle Amur Region	1	R7
12 Turi Rog	1	R10 ^a
13 Kavalerovo	1	R1
14 Pogranichnyy	2	R4, R4
15 Ussuriysk	2	R8, ^a R9 ^a
16 Vostok Bay	1	R1
17 Primorsky	1	R1 ^a
18 Russkyi Island	2	R3, R3, R3
19 Stenin Island	6	R2, R2, R2, R2, R2, R2
Siberia, Russia		
20 Boti, East Zabaikalye	1	S1 ^a
21 Chita, East Zabaikalye	1	R12 ^a
22 Adun-Chelon mnts., East Zabaikalye	8	R4, ^a R13, ^a S1, S1, S1, S1, S2, ^a S2
23 Tomsk	1	R12 ^a
24 Tuva	1	R12 ^a
25 Ultai	1	S3 ^a
Korea		
26 Yangyand	1	K2 ^a
27 Mt. Solak-san	1	K1 ^a
28 Munkyung	1	K1
29 Bongwha	1	K3
30 Milyang	1	K4 ^a
31 Mt. Chiri-san	1	K1
32 Piacol	1	K1

^aIndividual haplotype was subjected to 1143-bp analysis.

an automated method with a Dye Primer Cycle Sequencing Kit (ABI) and an automated sequencer (Model 373A; ABI).

Genetic distances (*d*) of Kimura (1980, Kimura's two-parameter) were calculated using the computer software program MEGA (Kumar *et al.*, 1993). Trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) using MEGA and by maximum parsimony (MP) using the software program PAUP*, Version 4.0b6 (Swofford, 2001). In the MP method, heuristic searches

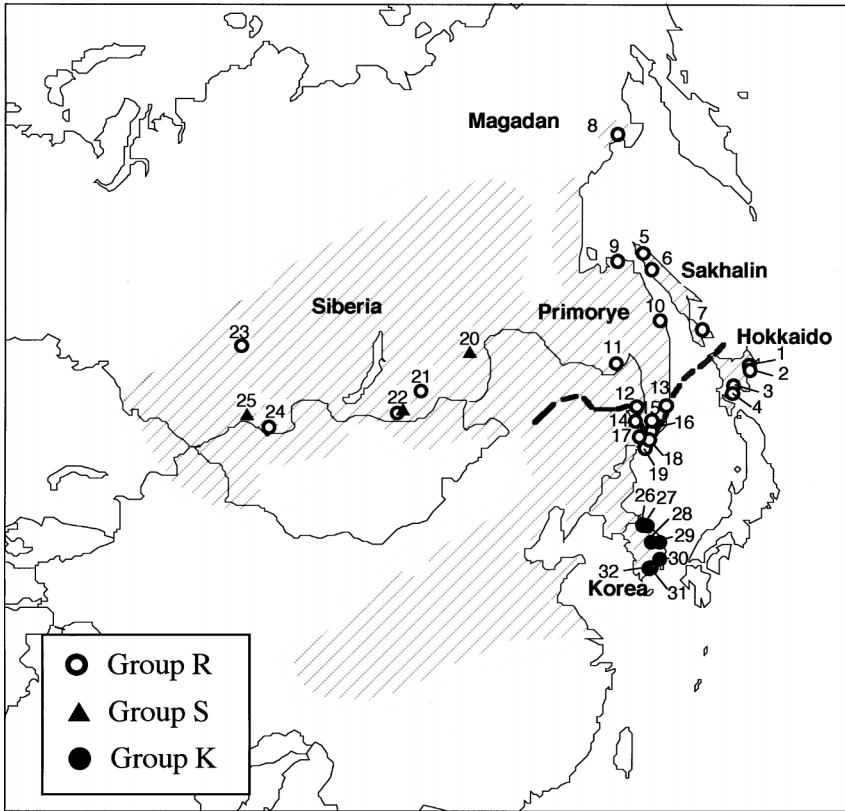


Fig. 1. Distribution of *A. peninsulae* and localities where samples were collected. Numbers assigned to localities are the same as in Table I. Types (see text): Korean, closed circle; Russian, open circle; and Siberian, triangle. The dotted line indicates the Last Glacial southern limit of the continuous permafrost zone (Ono, 1991).

were performed with 10 random addition replications with the tree bisection and reconnection option. Robustness of both NJ and MP phylogenies was assessed by the bootstrap analysis with 1000 resamplings. Nucleotide diversities (π ; p_i , average heterozygosity per nucleotide) were calculated using formula $\pi = n \sum x_i x_j \pi_{ij} / (n - 1)$, where n is the sample size, and x_i and x_j is the frequency of haplotype i and haplotype j , respectively (Nei and Jing, 1989).

RESULTS

Comparison of the sequences (402 bp) from 57 individuals revealed 23 haplotypes. Thirty sites were variable, and 31 substitutions could account for the differences among them. The genetic distances ranged from 0.0025 to 0.0308. To better

understand the relationships among the 23 haplotypes, we constructed an MP tree and traced the occurrence of 31 base substitutions along branches of the tree. Most of the substitutions can be assessed as single mutation events. Three base substitutions at sites 93, 303, and 321 (number refers to the nucleotide position from the starting point of the coding region of the *cyt b* gene) were plotted in two different branches. Regarding them as homoplasmy, we then constructed a network (Fig. 2). It was obvious that there was a clear tendency in the grouping of haplotypes into two clades. One clade included all haplotypes from the Korean Peninsula (here designated as K group), and also a portion of haplotypes from Siberia (S group; Fig. 1). Another clade included haplotypes from Primorye, Magadan, Hokkaido, and Siberia in part (R group). Haplotypes K1 and R1 connect the two clades of K/S and R by a smallest pass of five base substitutions and, therefore, are likely

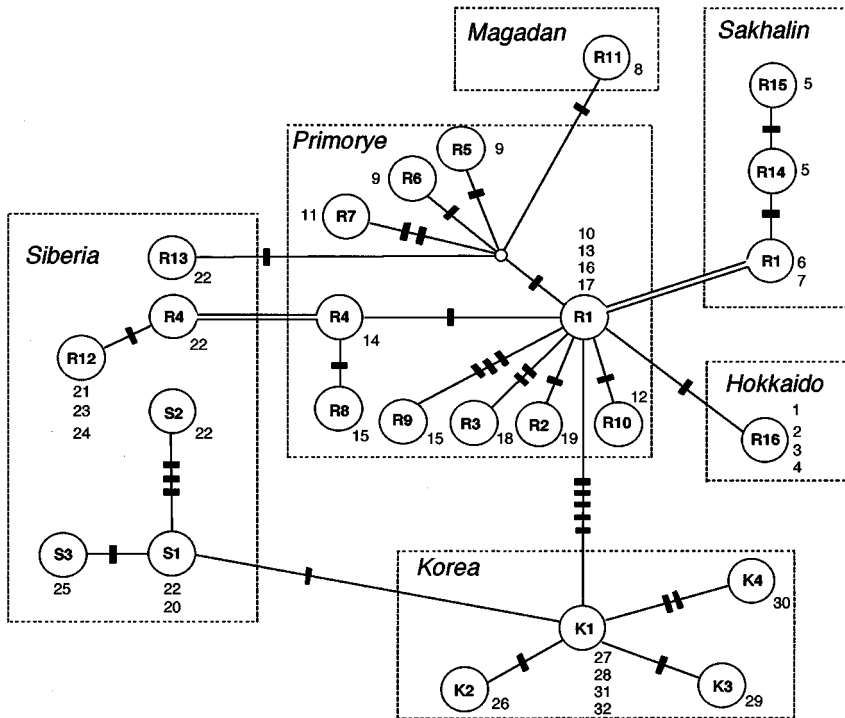


Fig. 2. A parsimonious tree of cytochrome *b* gene sequences (402 bp) of *A. peninsulae*. Base substitutions were plotted along the branches. The 23 haplotypes, each of which is represented by a circle, are divided into the six regions of Siberia, Primorye, Magadan region, Sakhalin, Hokkaido, and the Korean Peninsula, according to the original geographic source, with the numbers of collection localities listed in Table I. Twenty-three haplotypes detected could be subdivided into three main groups, Korean (K), Russian (R), and Siberian (S) types. The members of S (S1-3) and K (K1-4) were exclusively recovered from Siberia and Korea, respectively.

to retain ancestral sequences without accumulating further substitutions along the 402-bp region after differentiation at the five sites. The haplotypes K1 and R1 possessed 6 and 15 satellite isofemale lineages, respectively. Satellite haplotypes differ from K1 or R1 by one to four base substitutions.

The mtDNA haplotypes tend to be allocated to each of the six geographic regions of Hokkaido, Korea, Magadan, Primorye, Sakhalin, and Siberia. For example, the haplotypes R16 and R11 are each confined to Hokkaido and Magadan, respectively. The four types K1-4 are distributed in the Korean Peninsula. On the other hand, R1 was recovered from Primorye and the southern region of Sakhalin (Locality 5 in Fig. 1), while R4 were recovered both from Primorye (Locality 14) and Siberia (Locality 22).

We examined 13 individuals from six localities in Siberia (Localities 20–25; Fig. 1) as well as the boundary between Mongolia and Russia and found seven haplotypes. We note that haplotype groups of R and S are both present in Siberia. Eight individuals from three localities (Localities 20, 22, and 25) possessed haplotypes of the S group, in which sequences were more closely related to Korean individuals than to Russian ones. Such a tendency of admixture of mtDNA types in Siberia is also evident in the level of nucleotide diversity. The values are high in Siberia ($\pi = 0.0066$, $n = 13$ from Localities 20–25) and Primorye ($\pi = 0.0057$, $n = 12$ from Localities 10–19 excluding multiple individuals from tiny islands; $\pi = 0.0047$, $n = 8$ from Localities 12–17), low in Sakhalin ($\pi = 0.0012$; $n = 6$ from Localities 5–7) and the Korean Peninsula ($\pi = 0.0014$; $n = 7$ from Localities 26–32), and zero in Hokkaido and Magadan.

To examine the evolutionary aspects of the mtDNA mentioned above, we determined the entire *cyt b* gene (1143 bp) sequences for 25 representative individuals from 21 localities, covering 16 haplotypes of the 402-bp analysis (Table I). In the sequence comparison, 203 were variable, and 42 sites were parsimoniously informative. We then constructed NJ and MP trees using the sequence of *A. speciosus* as an outgroup (Fig. 3). We recovered 23 haplotypes, revealing two pairs of individuals that had the same sequence; one pair from western Siberia (Localities 23 and 24) and the other from Hokkaido (Localities 1 and 4). The mtDNA types diverged with substantial Kimura distances of 0.022 as the maximum. Four individuals with the R1 type from Sakhalin (Localities 6 and 7) and Primorye (10 and 17) were found to have different sequences in the 1143-bp analysis with Kimura distances of 0.0026–0.0062.

As in the 402-bp tree, the 1143-bp NJ tree gave a similar genealogical structure; two major clades (K/S and R) and several spatial-specific clades in each clade (Fig. 3). The K/S clade can be further subdivided into two clades, K and S, which represent haplotypes found in Korea and Siberia, respectively. The monophyly for each of Clades K and R was consistently supported by moderate bootstrap values (80–50%), irrespective of the algorithms and taxa of outgroup (*A. agrarius*, *A. semotus*, or *A. speciosus*), while the monophyly of Clade S was supported with

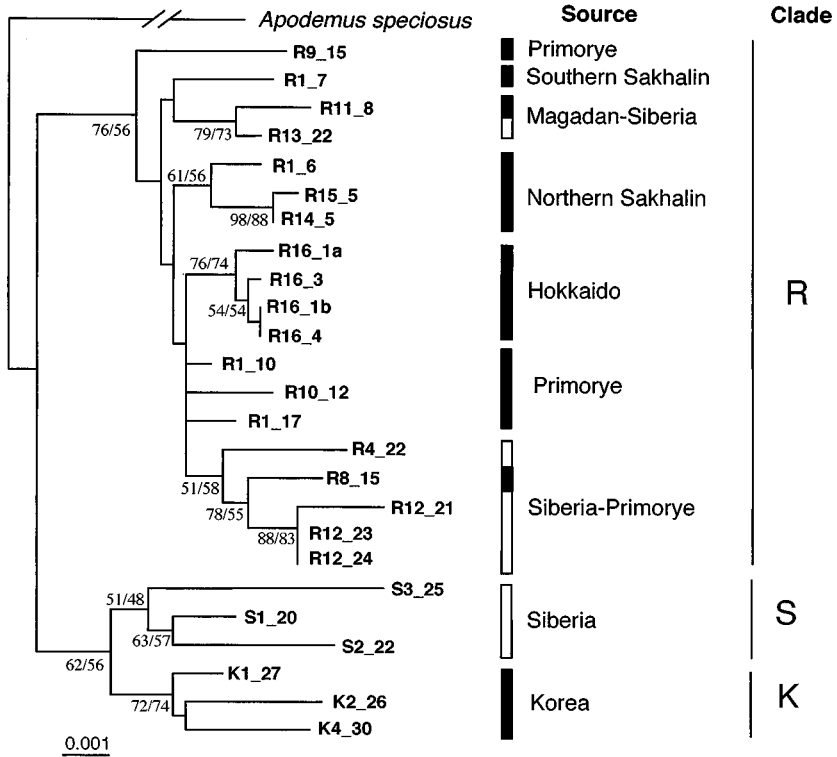


Fig. 3. Phylogenetic relationships among 25 haplotypes of *A. peninsulae* inferred from the mitochondrial cytochrome *b* gene (1143 bp). Haplotype labels are a combination of the 402-bp haplotype and collection locality as in Fig. 2. This tree was constructed by a neighbor-joining (NJ) algorithm and genetic distances computed by Kimura's two-parameter method in consideration of all codon positions and all substitutions, using the sequences of *A. speciosus* as an outgroup (Kimura, 1980). Bootstrap values (%) with 1000 replicates and high score (>50%) in NJ/MP trees are given at each node.

limited bootstrapping supports (about 50%), suggesting ancient lineage differentiation within Clade S.

Sequences for eight individuals from Siberia comprised three different types: Siberia-specific (S1-20, S2-22, and S3-25; Clade S), Primorye related (R12-21, R4-22, R12-23, and R12-24; Clade R), and Magadan related (R13-22; Clade R). The two related sequences from Magadan (R11-8) and Siberia (R13-22) differed slightly with $d = 0.0035$. In Sakhalin, on the other hand, geographic partition between northern (R14-5, R15-5, and R1-6) and southern (R1-7) parts can be seen in the mtDNA variation with maximum distance of 0.0071. Contrary to the result presented in Fig. 2, the haplotype from Locality 6 formed a clade with haplotypes from Locality 5.

DISCUSSION

From the present data, we can depict both temporal and spatial modes of the intraspecific variation of *cyt b* in *A. peninsulae*. The phylogenetic trees revealed the presence of three major clades, Clades R, K, and S in the haplotypes of *A. peninsulae* (Figs. 2 and 3), which are structured under the geographic architecture of the northern part of East Asia. Haplotype members of the last two clades are confined to the Korean Peninsula and Siberia, respectively. In contrast, the haplotypes of Clade R are distributed in both Siberia and the far eastern parts, including Magadan, Primorye, Sakhalin, and Hokkaido. These data give us a rationale for geographic subdivision into three parts, the Korean Peninsula, Siberia, and Primorye/Magadan/Sakhalin/Hokkaido when we consider the genetic structure of the mtDNA lineages in *A. peninsulae*. The geographic pattern of the mtDNA haplotypes also suggest that establishments of local lineages were mediated by a limited number of migrations across these regions of the Korean Peninsula and other parts of northeastern Asia during the long term of the evolutionary history. The highest level of Kimura distance among these haplotypes is 0.022. If we take into account the widely accepted divergence rate of mtDNA, about 0.02 per million years (Brown *et al.*, 1979), the genealogical diversity is considered to have accomplished in the last one million years.

The Korean population of *A. peninsulae* has an mtDNA lineage distinct from those of Primorye, despite a relatively close geographic disposition and close affinity in the patterns of B-chromosomes (Koh, 1986) and morphology (Musser *et al.*, 1996; Vorontsov *et al.*, 1977). Substantial difference among the *cyt b* sequences from Korea ($d = 0.006\text{--}0.008$) suggests long-term persistence of the *A. peninsulae* population in this area. In agreement, the Korean red-backed vole (*Eothenomys regulus* or *Clethrionomys regulus* according to taxonomists; see Musser and Carleton, 1993) from the Korean Peninsula is known to be distinct from the corresponding Russian species, *C. rufocanus* based on morphology (Kaneko, 1990), differences in *cyt b* (Suzuki *et al.*, 1999) and features of the Y chromosome (Iwasa *et al.*, 1999). In the case of *A. agrarius*, as another example, field mice from Korea exhibit specific patterns in the *cyt b* gene sequences which differ from those of Russian individuals from Primorye (Han *et al.*, unpublished data). Thus, these available data demonstrate that the Korean Peninsula has the potential to maintain its own populations of small rodents through substantially long-term evolutionary time, including the Quaternary glacial ages. The notion that the Korean forests could act as a refugium also accords well with the paleogeographical data for East Asia (Nazarenko, 1990; Tsukada, 1985).

The geographic partition of the mtDNA genealogy of *A. peninsulae* can be seen among populations of Primorye, Siberia, Magadan, Sakhalin, and Hokkaido (Figs. 2 and 3). Such a tendency in the mtDNA variation has been seen before in small mammals, such as red-backed voles, *Clethrionomys rufocanus* (Iwasa *et al.*, 2000; Suzuki *et al.*, 1999; Wakana *et al.*, 1996) and *C. rutilus* (Iwasa *et al.*,

2002; Suzuki *et al.*, 1999). Thus, the geographic configuration of the northern part of East Asia itself can be considered to provide important factors for the generation of genetic variation in small mammals. In particular, insular regions of Sakhalin and Hokkaido appear to provide such functions, as initiation and maintenance of genetic divergence. Moreover, it has been suggested that Hokkaido assisted a speciation process in rodents, as a consequence of persistence of the genetic diversity for a long time, through phylogenetic analyses of *C. rex* endemic to Hokkaido and the southernmost tip of Sakhalin (Iwasa *et al.*, 2000; Wakana *et al.*, 1996). From our present data of *A. peninsulae*, it is also evident that subdifferentiation has occurred between Northern Sakhalin and Southern Sakhalin (Fig. 3).

The extent of the intraspecific variation among the Russian Far East, Sakhalin, and Hokkaido populations tends to differ among species. Intraspecific variation is low in *A. peninsulae*, red foxes (Tsuda *et al.*, 1997), and sables (Hosoda *et al.*, 1999) and high in red-backed voles (Iwasa *et al.*, 2000, 2002; Suzuki *et al.*, 1999). In the case of vole species, Hokkaido retains the old lineage of mtDNA as observed in *C. rufocanus* and *C. rutilus* (Iwasa *et al.*, 2000; Suzuki *et al.*, 1999). This simply implies that the extant mtDNA lineage of *A. peninsulae* arises from that settled in Hokkaido rather recently. Then there are two possible explanations for why the Hokkaido population of *A. peninsulae* showed low levels of genetic diversity. One is the recent colonization by *A. peninsulae* of Hokkaido, and the other is the recent gene flow of mtDNA into the Hokkaido population. Allozyme data seem to support the former possibility. Our preliminary study indicated that populations of Hokkaido share quite similar genetic properties with the Sakhalin population in allozyme variation and are monomorphic on the basis of 22 protein loci, including the Tf locus which is polymorphic in populations of the Russian Far East and Sakhalin (Pavlenko, 1989; Pavlenko *et al.*, unpublished). It has been shown that the populations of the Russian Far East and Sakhalin retain a very similar genetic constitution with Nei's genetic distances of 0.003–0.014 (Pavlenko, 1994a,b; Pavlenko and Kartavtseva, 1998). These extant patterns of allozyme differentiation have been explained by a rapid northward expansion of *A. peninsulae* over the Russian Far East, Sakhalin, and Hokkaido from a single late glacial refuge during the Holocene (Pavlenko, 1989, 1994a,b).

On the other hand, recent karyological investigation in *A. peninsulae* showed that there is substantial geographic variation in the profile of B-chromosome; B-chromosomes are often seen in Primorye–Hokkaido but scarcely in Sakhalin (Kartavtseva *et al.*, 2000). The present data on the *cyt b* gene may provide insight into the evolution of *A. peninsulae* with special reference to the colonization of such founder populations, but further study with nuclear markers would be needed to understand actual situation of the intraspecific genetic differentiation.

From these considerations, the region of Primorye would have played important roles in the mtDNA diversification in *A. peninsulae*. Primorye shows a high genetic diversity, and the neighboring populations of Sakhalin and Hokkaido now

possess descendant lineages of mtDNA dispersed from Primorye. The lineage dispersal from Primorye would have occurred, probably because of high individual density compared to the other regions. The question next is how the Primorye and Siberia populations associated in the mtDNA lineage differ. The two geographic areas appear to differ in composition of haplotypes of Clades R and S. Since the members of Clade S are endemic to Siberia and there is considerable genetic diversity within the clade, it would be probable that the mtDNA lineage of Clade S emerged in Siberia after splitting from the lineage of K (Fig. 3) and remained in Siberia without dispersing to other geographic area. If this is true, the other haplotypes of Clade R in Siberia can be considered to be descendants of those migrating from other parts such as Primorye and Magadan relatively recent in evolutionary time.

The present data, moreover, provides an important perspective of the evolution of *A. peninsulae* in the Quaternary geological changes: The presence of three distinct lineages in northern East Asia exhibiting substantial geographic allocation suggests persistence of local populations in Siberia, Primorye, and Korea during a considerable part of the ice ages (Fig. 3). The northern limit of the distribution of the extant populations of *A. peninsulae* is likely to be defined by certain environmental conditions such as glaciation. In fact, the southern limit of the distribution of the permafrost at present (Ono, 1991) may fit with the northern limit of *A. peninsulae*. Because in the Last Glacial the southern border of the permafrost is thought to have reached the southern tips of Primorye and Sakhalin (Fig. 1; Ono, 1991), we initially supposed that *A. peninsulae* was forced to withdraw to the most southern part of Primorye during the Last Glacial. However, our present data demonstrate that populations of field mice have been sustained in Primorye and even in Siberia during the last one and a half million years. Therefore, the three geographic regions of Primorye, Siberia, and the Korean Peninsula can be noted as refugia that persisted through several rounds of the Quaternary glacial ages.

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