

Mitochondrial Genetic Diversity and Phylogenetic Relationships of Siberian Flying Squirrel (*Pteromys volans*) Populations

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Abstract: Siberian flying squirrel, an endangered species in South Korea, is distributed through major mountain regions of South Korea. The number of Siberian flying squirrel (*Pteromys volans*) in South Korea has decreased and their habitats are fragmented and isolated because of anthropogenic activities. So far no molecular genetic data has, however, been available for their conservation and management. To obtain better information concerning genetic diversity and phylogenetic relationships of the Siberian flying squirrel in South Korea, we examined 14 individuals from South Korea, 7 individuals from Russia, and 5 individuals from northeastern China along with previously published 29 haplotypes for 1,140 bp of the mtDNA cytochrome *b* gene. The 14 new individuals from South Korea had 7 haplotypes which were not observed in the regions of Russia and Hokkaido. The level of genetic diversity (0.616%) in the South Korean population was lower than that in eastern Russia (0.950%). The geographical distribution of mtDNA haplotypes and reduced median network confirmed that there are three major lineages of Siberian flying squirrel, occupying; Far Eastern, northern Eurasia, and the island of Hokkaido. The South Korean population only slightly distinct from the Eurasia, and eastern Russian population, and is part of the lineage Far Eastern. Based on these, we suggest that the South Korean population could be considered to belong to one partial ESU (Far Eastern) of three partial ESUs but a different management unit. However, the conservation priorities should be reconfirmed by nuclear genetic marker and ecological data.

Key words: Siberian flying squirrel, cytochrome *b* sequence variation, genetic diversity. Evolutionary significant unit (ESU)

Introduction

The identification of genetically distinct local populations and appropriate taxonomic units for protection and management is a requisite for the conservation of biological diversity (Allendorf and Luikart, 2007). In addition, an understanding on genetic diversity of a population or species of concern is also indispensable. Genetic diversity which reflects the evolutionary potential to adapt to ever-changing environment is a major issue of current conservation biology (Frankham et al., 2002), and the loss of genetic diversity is an important emphasis of conservation genetics (Avice, 2004). Commonly, critically endangered species or population tends to experience decrease of population sizes, often leading to loss of genetic diversity. Genetic analysis can also assist in providing accurate taxonomical information that can aid in implementing adequate restoration programs. The restoration project of Asiatic black bear (*Ursus thibetanus*) in South Korea is a good example. Phylogenetic analysis of eastern Asian black bear populations using mitochondrial markers helped to identify the appropriate conservation units based on evolutionary significant units before reintroducing the Asiatic black bear from other regions (Hong, 2005).

The Siberian flying squirrel, *Pteromys volans*, is a nocturnal, arboreal rodent that inhabits spruce-dominated boreal forest (Selonen et al., 2005), throughout the whole coniferous forest zone of Eurasia from Finland and the Baltic Sea to eastern Siberian and Korean Peninsula, Sakhalin Island of Russia, and Hokkaido Island of Japan (Nowak, 1999). In South Korea, the Siberian flying squirrel occurs in extreme north-eastern boreal forests as well as

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southward in suitable habitats along the Tae-bak Mountain range (Won and Smith, 1999). Their habitat has been diminished by the clearing of forests during 1960s and 1970s as well as dam construction and from the use of pesticide to control a worm, which resulted in a severe shrinkage in the number of populations and range as well as habitat fragment (Won and Smith, 1999). Moreover, the Siberian flying squirrel in South Korea has been used in oriental medicine in some rural areas (Yoon et al., 2004). Consequently, the Siberian flying squirrel in South Korea was classified as endangered species by Ministry of Environment in South Korea and a Natural Monument species (No. 328) by Cultural Heritage Administration of Korea.

There have been several ecological studies on the Siberian flying squirrel in other countries, especially Finland (Hanski et al., 2000; Reunanen et al., 2002; Timm and Kiristaja, 2002; Selonen and Hanski, 2004; Selonen and Hanski, 2006; Hurme et al., 2007), but molecular genetic studies on the Siberian flying squirrel have been less common (e.g. a phylogeographic study - Oshida et al., 2005; isolation and characterization of microsatellite loci - Painter et al., 2004; microsatellite variation - Selonen et al., 2005). Among these, Oshida et al. (2005) reported the phylogeographic status of *P. volans* from broad regions, but excluding the Korean peninsular and China. Recently, the study on Siberian chipmunk, having a similar distribution with Siberian flying squirrel, using the mtDNA cytochrome *b* gene revealed that the Siberian chipmunk in northeastern China is highly diverged even though the specimens were collected in only one regions, Heilongjiang, China (Lee et al., in preparation). Besides, the Siberian chipmunk in South Korea is extremely different to the one in Russia and China (Lee et al., in press). The result of the codistributed species displayed that the urgent necessity to molecular genetics researches on genetic diversity and phylogeographic study with the Siberian flying squirrel, which would help better understand evolutionary history and population composition of small mammals in Eurasia as well as South Korea.

In the present study, the entire sequence (1,140 bp) of mtDNA cytochrome *b* gene was used as a genetic marker in order to compare with existing data. We evaluated genetic diversity and phylogenetic relationships of the Siberian flying squirrel in South Korea and northeastern China along with previously published data (Oshida et al., 2005). Our results will assist in the development of a conservation or management strategy for this endangered species.

MATERIALS AND METHODS

Sampling

A total of 27 specimens were taken from the Conservation Genome Resource Bank for Korean Wildlife (CGRB), including one blood sample, one liver sample, 14 dried skin samples, and 11 muscle samples. DNA extraction of the one dried skin sample (CGRB3507) failed because of severe degradation. Therefore, a total of 26 specimens were used for this study (Table 1). Seven specimens were collected from two geographic localities of Russia and 14 specimens were from two geographic localities of South Korea; Jellanam-do and Gyeonggi-do (Fig. 1). Five specimens were from Heilongjiang Province, China. The specimens were from carcasses of road-kills or death by unknown causes found by local people or field researchers and Russian and Chinese specimens were trapped. The collecting localities of our specimens are very biased due to difficulty in collecting them and protection under the law. We obtained the sequences of complete cytochrome *b* gene for 29 haplotypes of *P. volans* and 2 individuals of *P. momonga* from GenBank, for use as outgroups (AB164478, AB164478, AB164648-AB16476: Oshida et al., 2005).

DNA isolation, amplification and sequencing

Genomic DNA was extracted from the tissue and dried skin using the QIAamp tissue kits (Qiagen, USA). Blood DNA was extracted using the Blood Genomic DNA Isolation Kit (CoreBioSystem Co.). The procedures followed the instruction of each Kit. To amplify the complete mitochondrial cytochrome *b* gene, one pair of primer was used; L14724

Table 1. Sample sources, number of individuals, clades, and haplotypes

Country	Collecting locality	No. of Individual	Clade	Haplotype	CGRB ID
South Korea	Gapeong, Gyeonggi-do	1	Far Eastern	PV30	366
South Korea	Pochen, Gyeonggi-do	1	Far Eastern	PV31	3020
South Korea	Gurye, Jeollanam-do	11	Far Eastern	PV33, PV34, PV35, PV36	1190, 1206, 1498, 1521, 1530, 1682, 1781, 2089, 2135, 2493, 3708
South Korea	Suncheon, Jeollanam-do	1	Far Eastern	PV32	1348
China	Heilongjiang province	5	Far Eastern	PV41, PV42, PV43, PV44, PV45	5938, 5939, 5940, 5941, 5942
Russia	Chitinskaja oblast	4	Northern Eurasia	PV37, PV38, PV39	3148, 3149, 3150, 3151
Russia	Kirov province	3	Northern Eurasia	PV25*, PV40	3051, 3052, 3508

*This haplotype corresponds to the haplotype of previous study, Oshida et al., (2004)

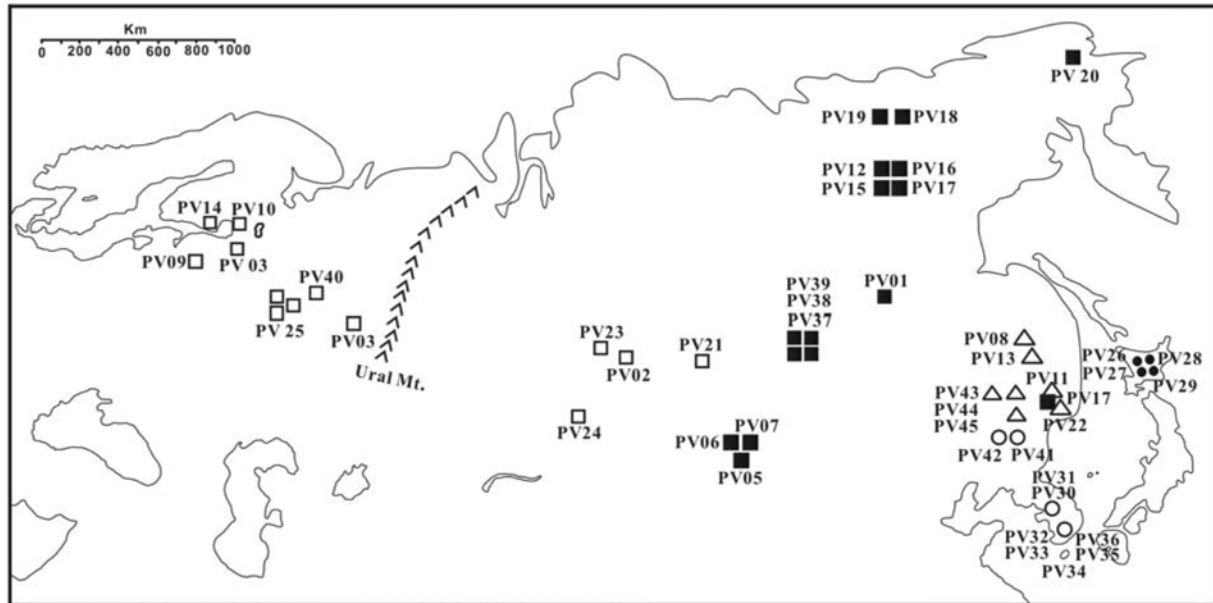


Fig. 1. Map of distribution and sampling locations in the present study. Black circles (●), Black squares (■), open triangles (△), open squares (□), and open circles (○) represent the subclades: Hokkaido, central Siberian part of Russia, eastern Russia, northwestern Eurasia, and South Korea.

5'-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3' and H15915 5'-AAC TGC AGT CAT CTC CGG TTT ACA AGA C-3' (Kocher et al., 1989). The final reaction concentration of PCR mixture was as follows: each dNTP at 2.5, 0.5 mM of each primer, 1 unit of i-star Taq polymerase (iNtRON Biotechnology), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 20-50 ng of genomic DNA was used as a template. PCR amplification was carried out in a total volume of 20 µL. An initial denaturing step at 94°C for 5 min was followed by 35 cycles at 94 for 45 s, 45 for 60 s, 72°C for 90 s and, a final run at 72°C for 7 min completed program. PCR products were electrophoresed through a 1.5% agarose gel, stained with ethidium bromide, and examined using ultraviolet light. Successful amplicons were purified with the Zymoclean Gel DNA Recovery Kit (Zymo research, USA). Primer L14724, L15162 (Irwin et al., 1991), and H15915 was used to sequence the complete cytochrome *b* gene with an Applied Biosystems 3730 XL DNA sequencer according to manufacturer's instructions. Sequence data generated in this study have been deposited to GenBank (Accession Nos. EU919142-EU919160 and FJ360736-FJ360740).

Data analysis

Multiple sequence alignments were carried out using Clustal_X (Thompson et al., 1997) and edited by BIOEDIT (Hall, 1999). Phylogenetic relationships among haplotypes of cytochrome *b* gene were estimated using maximum parsimony (MP) by PAUP version 4.0 beta 10 (Swofford, 2001). The maximum-parsimony (MP) tree was constructed with the heuristic search algorithm with tree-bisection-

reconnection in PAUP. To assess nodal support, bootstrapping (Felsenstein, 1985) was performed with 100 replicates in the MP analysis. The strict consensus was calculated for all most parsimonious trees and taken as the best phylogeographic hypothesis for Siberian flying squirrel. Reduced median network (Bandelt et al., 1999) was drawn using the program Network 4.0 (<http://www.fluxus-engineering.com>) to investigate the possible relationships among haplotypes of Siberian flying squirrels. The number of polymorphic sites, haplotype diversity (*h*) and nucleotide diversity (π) were calculated using DNASP 4.10 (Rozas et al., 2003) and ARLEQUIN ver 3.01 (Excoffier et al., 2005). Pairwise comparison of Φ_{st} values among subclades, using haplotype data and pairwise difference, was carried out with ARLEQUIN ver 3.01. The pairwise sequence difference among subclades was calculated by Kimura two-parameter using MEGA version 4 (Tamura et al., 2007).

RESULTS

Mitochondrial cytochrome *b* variability

A complete fragment (1,140 bp) of mitochondrial cytochrome *b* gene of 26 individuals from 5 geographic populations was successfully determined and analyzed together with 29 previously published Siberian flying squirrel haplotypes from GenBank (Accession Nos. AB164478, AB164479, and AB164648-AB164676). There were no indels (insertions and deletions) in complete cytochrome *b* sequences. A total of 45 haplotypes were defined by 114 polymorphic sites, of which 57 were parsimony informative sites (94 transitions and 21 transversions) (Fig. 2 and Table 2). The overall

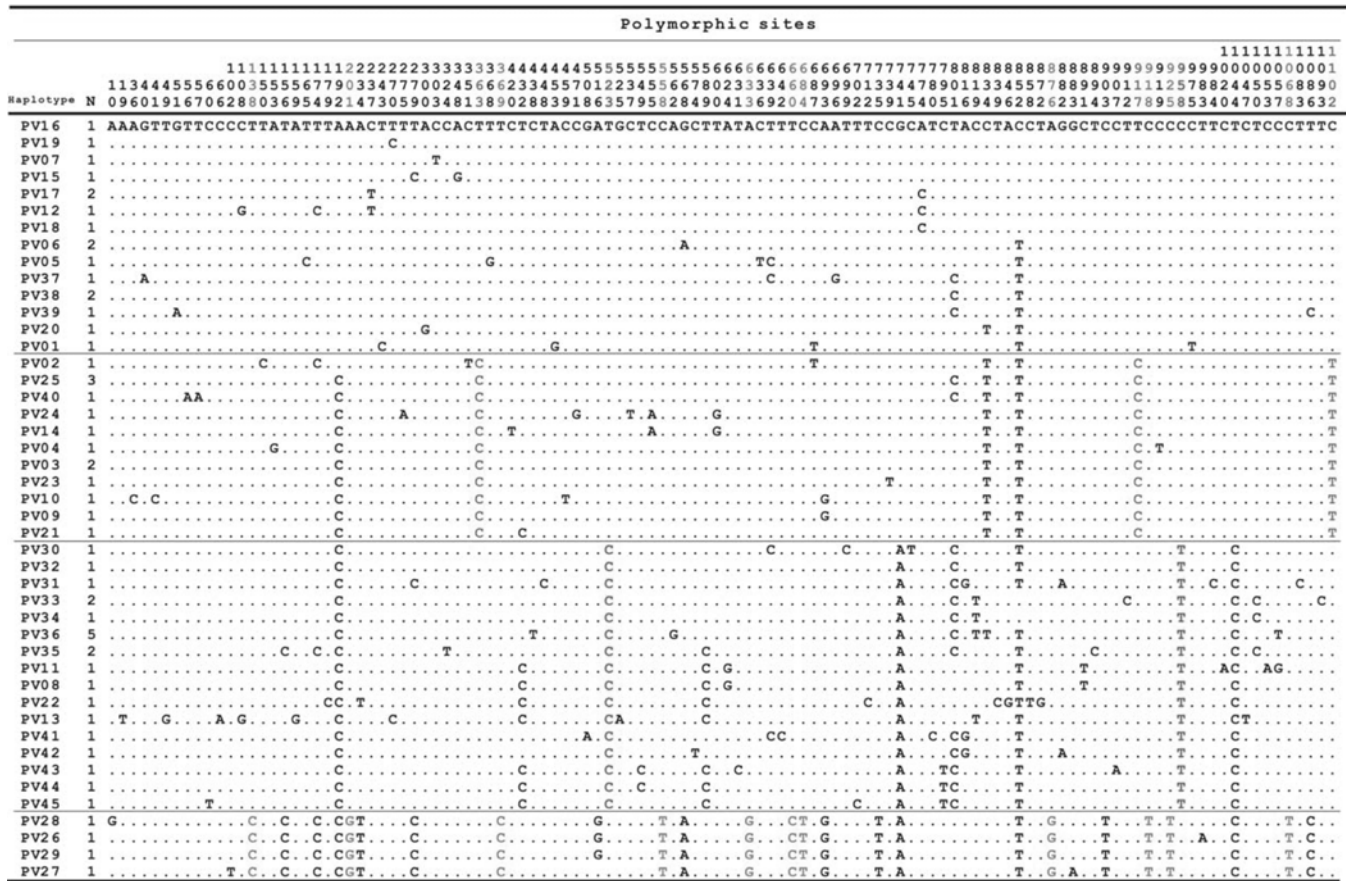


Fig. 2. Alignment of mtDNA cytochrome *b* haplotypes. Only variable positions are shown.

haplotypes diversity and the nucleotide diversity were 0.985 ± 0.007 and $1.164\% \pm 0.589$, respectively. The level of diversity within the South Korean population (nucleotide diversity, $\pi = 0.616\% \pm 0.070$) is slightly higher than that of the lineage northern Eurasia ($\pi = 0.591\% \pm 0.318$) and much lower than eastern Russia ($\pi = 0.950\% \pm 0.246$). However, the level of diversity of the Siberian flying squirrels within South Korea is much lower than that of the Siberian flying squirrels ($\pi = 1.169\% \pm 0.135$) from the rest of the regions we examined except for South Korea. Our results suggest

that nucleotide diversity within populations is small relative to among population nucleotide diversity (Table 2). The sequences in South Korean population examined here were location-specific. The largest haplotype group (PV36) consisted of 5 individuals from South Korea, 7 haplotypes including 2 individuals (PV03, PV06, PV17, PV33, and PV38) and 3 individuals (PV25 and PV35) (Fig. 2).

Haplotype distribution and sequence alignment reveals the Siberian flying squirrel in Hokkaido Island of Japan was highly divergent to other regions. Aligned sequences

Table 2. Summary of sample sizes (N), number of haplotypes (n), nucleotide diversity (π), haplotype diversity (h), polymorphic site, singleton, parsimony informative sites for the specimens used in this study

	N	Haplotypes (n)	h (SE)	π (% \pm SE)	Polymorphic sites	Parsimonious-informative sites	Singletons
Overall	58	45	0.988 (0.007)	1.164 (0.589)	114	57	57
Hokkaido	4	4	1.000 (0.177)	0.219 (0.067)	5	0	5
Northern Eurasia	31	25	0.986 (0.012)	0.591 (0.318)	47	15	32
Eastern Russia	4	4	1.000 (0.177)	0.950 (0.246)	21	19	2
China	5	5	1.000 (0.126)	0.614 (0.124)	15	5	10
South Korea	14	7	0.846 (0.074)	0.616 (0.070)	23	14	9
Far Eastern*	23	16	0.945 (0.034)	0.804 (0.428)	54	23	31

Standard deviations for h and π are in brackets. * Lineage Far Eastern includes South Korea, China and eastern Russia.

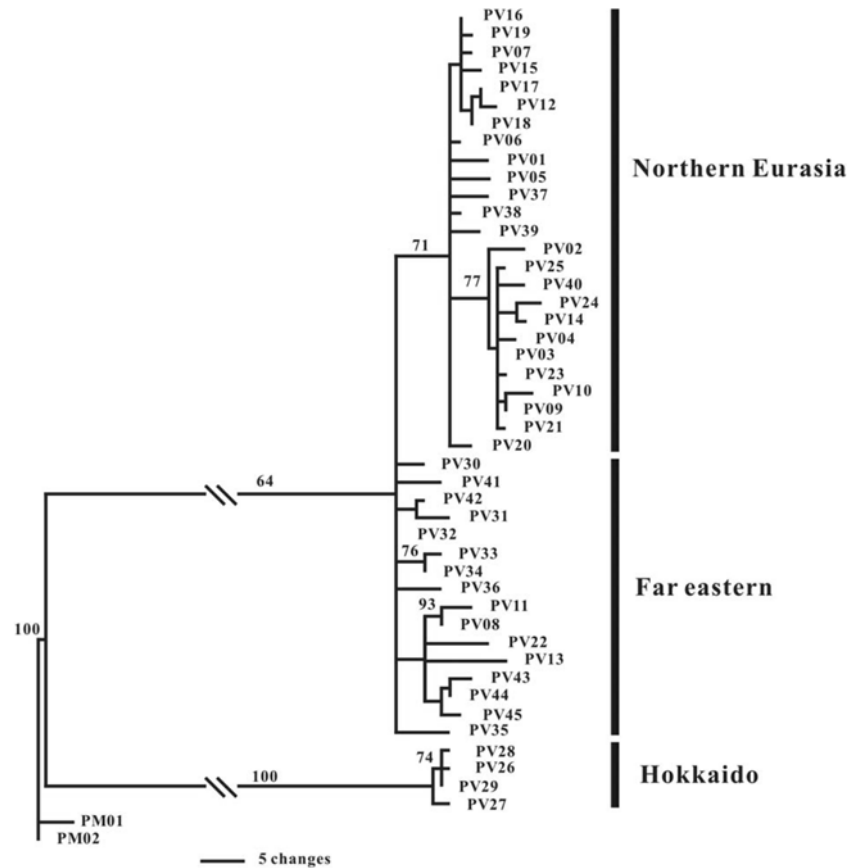


Fig. 3. Phylogenetic relationships among 45 haplotypes of Siberian flying squirrel mitochondrial cytochrome *b* gene. Numbers at the major clades denote the bootstrap values.

(Table 2) showed 11 single nucleotide polymorphisms (SNPs) which could be considered as diagnostic sites for distinguishing subspecies affiliation in Siberian flying squirrel. The sequences of Far Eastern lineage, composed of South Korean, Chinese and eastern Russian population, displayed two SNP differences (Table 2: positions 523 and 958) distinguishing it from lineages Hokkaido and northern Eurasia. Among subclades of Siberian flying squirrel, northwestern Eurasia subclade displayed three SNP differences (Table 2: positions 363, 917, and 1102). Overall, the pattern of sequence alignment revealed each subclade was divergent by isolation by distance.

Phylogenetic relationships

The ML tree (Fig. 3) constructed with 45 haplotypes clearly elucidated that there were three major lineages corresponding with the previous result of Oshida et al. (2005) and two subclades in lineage Far Eastern were found, which comprised the South Korean, the eastern Russian, and the Chinese population. However, there were no shared haplotypes among three regions. Seven individuals from two different localities of Russia fit into northern Eurasia lineages which are coincident with the result of Oshida et al. (2005). The reduced median network of 45 haplotypes

of Siberian flying squirrel also confirmed this strong phylogeographic structure (Fig. 4) consisting of lineages Far Eastern, northern Eurasia, and Hokkaido. There were also 2 subclades in lineages Far Eastern and northern Eurasia, respectively. The South Korean haplotypes were part of the Far Eastern lineage but the South Korean haplotypes cluster together and separated from the haplotypes of eastern Russia (Fig. 4). Of five individuals from Heilongjiang, China which resulted in five haplotypes: two haplotypes (PV41 and PV42) grouped with South Korean population while three haplotypes (PV43, PV44, and PV45) clustered together with eastern Russian population. The average pairwise divergence rate among haplotypes was 1.4% between the lineage Far Eastern and others, compared with 0.8% within the lineage Far Eastern and 0.6% within the lineage northern Eurasia.

DISCUSSION

There are three species in Sciuridae in South Korea; Siberian chipmunk, Eurasian red squirrel, and Siberian flying squirrel (Yoon et al., 2004). Of them, the Siberian flying squirrel is designated as an endangered species and as a natural monument in South Korea. On the other hand,

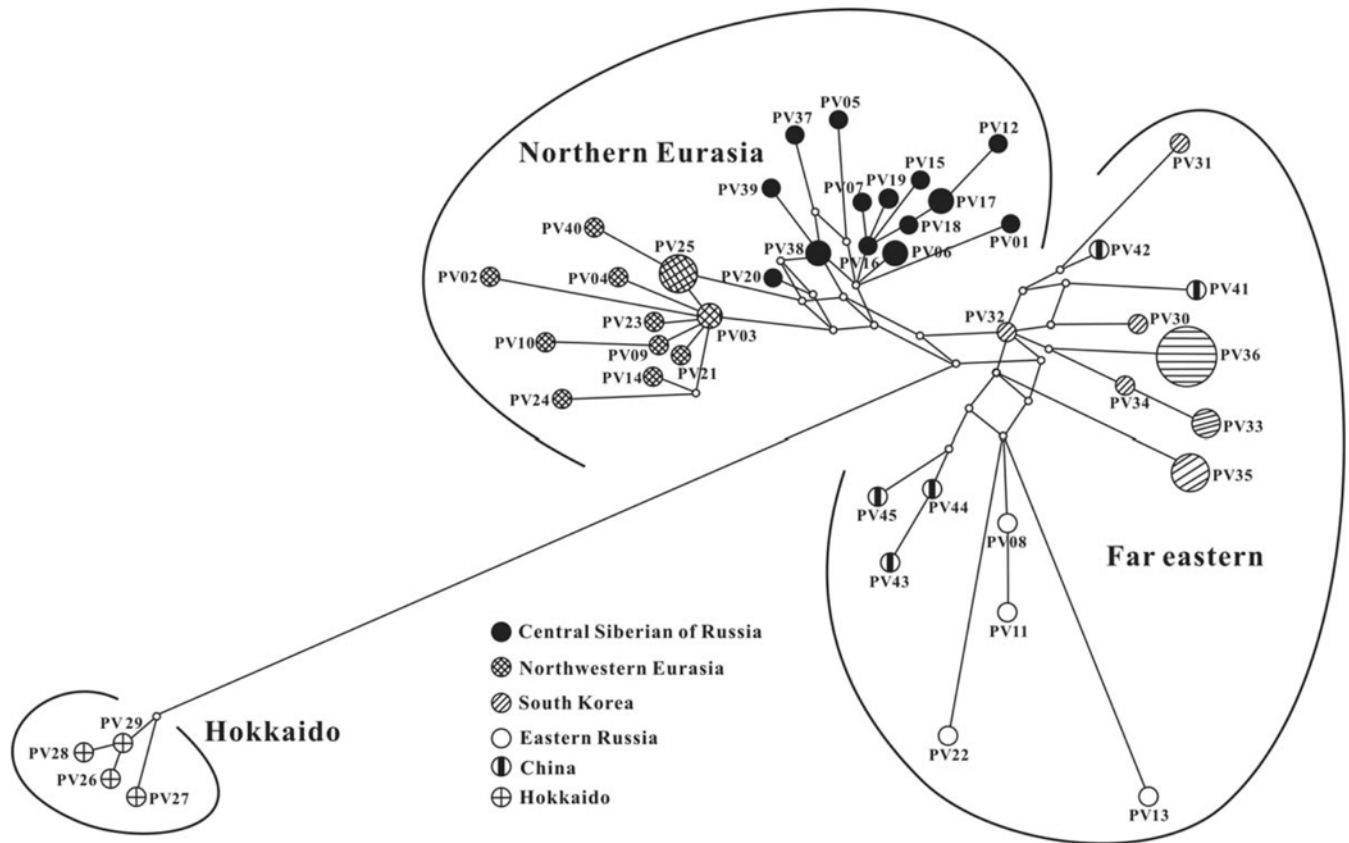


Fig. 4. Reduced median network of the 45 cytochrome *b* haplotypes of the Siberian flying squirrel (*Pteromys volans*). The size of the node indicates the relative frequency of the corresponding haplotype in the whole data set.

the Siberian chipmunk and Eurasian red squirrel are very common in South Korea. In particular, the Eurasian red squirrel on farms is culled by hunters for pest control during the harvest season of pine nuts (Yoon et al., 2004). Recently, molecular genetic studies of the Siberian chipmunk have revealed that there are large genetic distinction and strong phylogeographic architecture between the Siberian chipmunk from South Korean peninsular and Russia - northeast China using mtDNA cytochrome *b* gene (Lee et al., in press). However, no population structure and genetic differentiation of Eurasian red squirrel in eastern Russia, northeastern China, and South Korea were found when using same genetic marker (data not shown).

Oshida et al. (2005) in a phylogeographic study of the Siberian flying squirrel in Russia and Hokkaido, Japan demonstrated refugia theory in arboreal small mammal of Eurasia in terms of the findings, three major lineages, Far Eastern, northern Eurasia, and Hokkaido and the level of genetic diversity. Our analysis with the additional South Korean and northeastern Chinese sample confirmed the clear maternal differentiation among the three regions. Most Siberian flying squirrel haplotypes were unique to one of the geographical populations examined (Fig. 2 and 4), and genetic variation was structured geographically

except for Chinese haplotypes. South Korean populations grouped with the Far Eastern haplotype. However, within the Far Eastern group, significant population differentiation was observed between eastern Russia and South Korea (Fig. 4 and Table 3). No shared haplotypes were found between eastern Russia and South Korea. These results suggest that there is some degree of spatial genetic structure within the Siberian flying squirrel in these regions and limited maternal dispersal of individuals among local populations. This result was supported by phylogenetic trees, reduced median network, and Φ_{st} value (Table 3) as well.

The cytochrome *b* haplotype distribution of the Siberian flying squirrel didn't reveal a large amount of phylogeographical structure. In particular, the Ural Mountain doesn't seem to have been a genetic barrier (Fig. 1) to the Siberian flying squirrel even though the species is ecologically associated with a boreal habitat affected by glaciations during Ice ages (Oshida et al., 2005). In addition, the study demonstrated that the Magadan region, the northeastern part of Russia, has a unique genetic clade among some rodents (Lee et al., in press; Serizawa et al., 2002; Iwasa et al., 2002). However, our study showed a different pattern of genetic differentiation as no genetic barriers were observed in

Table 3. Pairwise Φ_{st} values based on molecular distance (pairwise difference) among subclades (below diagonal) and sequence divergence of the Siberian flying squirrel among subclades (above diagonal)

	NWE	CSR	SK	ER	Ho
NWE		0.008	0.013	0.015	0.026
CSR	0.494**		0.011	0.012	0.024
SK	0.534**	0.569**		0.010	0.026
ER	0.587**	0.606**	0.276**		0.027
Ho	0.860**	0.848**	0.779**	0.778*	

1. * and **, respectively significant at 0.005 and 0.001 level.

2. SK: South Korea subclade, ER: eastern Russia subclade, NWE: northwestern Eurasia subclade, CSR: central Siberian part of Russia subclade, Ho: Hokkaido clade.

Magadan regions and north Mongolia/Transbaikalia regions, the southern part of Russia (Fig. 1) despite their relatively remote geographic separation. This suggests that Siberian flying squirrel went through a different evolutionary history than similarly distributed species and/or that the Siberian flying squirrel could have migrated to broad regions and has low substitution rate of mitochondrial DNA like red wood ants (Goropashnaya et al., 2004).

Among rodents, there have been several studies revealing that the genetic differentiation between Korean peninsular and other regions including Hokkaido of Japan, Sakhalin of Russia and Siberia, with a big or moderate genetic divergence (Serizawa et al., 2002; Yasuda et al., 2005; Lee et al., in press). Our study is congruent with this pattern even though it's shallow and considered to be a sublineage of the lineage Far Eastern. It is likely to be difficult to figure out population history and substructure in South Korea because the samples used in this study are very biased and not large enough to test. However, the study concerning population structure of Siberian chipmunk clarified that there are three phylogroups in South Korea (Lee et al., in press). In terms of conservation aspects, future studies should include unbiased sampling from overall distribution range of the species to assess population history and substructure, which would be very helpful in formulating a conservation strategy of the Siberian flying squirrel.

The fourteen individuals in South Korea examined here resulted in 7 haplotypes and the nucleotide diversity was 0.616%. The sample from eastern Russia and China is small (4 haplotypes and 5 individuals, respectively), and so sampling effects could be considered. When comparing the nucleotide diversity which is relatively less affected by the population size (Graus and Li, 2000), the level of genetic diversity in South Korean population was lower than that (0.950%) in eastern Russia (Table 2). However, the level of genetic diversity in South Korea was comparable to that in northern Eurasia (Table 2). High genetic diversity suggests that this species probably had large effective population sizes in their recent history (Christopher et al., 2003). Eastern Russia has been considered to be a well protected region, with large protected regions, such as the Lazovsky

reserve. By contrast, in South Korea there have been the mounting pressures for development, such as the construction of highway and the destruction of forests by forest fire and construction of entertainment facility, which leads to the habitat destruction as well as habitat fragmentation. These effects might be one of reasons to show the low level of genetic diversity in South Korea. Reforestation can increase gene flow and reestablish levels of genetic variations (Paulauskas et al., 2006).

Finding appropriate conservation units for endangered and threatened species is very crucial (Allendorf and Luikart, 2007). Until now, the concept of the Evolutionary Significant Unit (ESU) has been debated since it was conceptualized in 1986 as a conservation unit under the species level (de Guia and Saitoh, 2007; Crandall et al., 2000; Fraser and Bernatchez, 2001; Bowen, 1998). In recent, de Guia and Saitoh (2007) proposed the use of full ESU and partial ESU designations according to which data (neutral genetic variation and adaptive variation) was used for assigning population distinctiveness. In this study, we collected only neutral genetic variation such as molecular genetic data and there is currently very few ecological, life history, or quantitative genetic data available for the Siberian flying squirrel. Therefore, only partial ESU can be recommended here. The definition of partial ESU is almost comparable to Moritz's definition of ESU (Moritz, 1994) that populations are reciprocally monophyletic for mtDNA alleles and have shown significant divergence of allele frequencies at nuclear loci. Based on Moritz's definition, the data for the Siberian flying squirrel suggest that the three monophyletic mtDNA lineages could be considered to be conservation units (Far Eastern, northern Eurasia, and Hokkaido). However, the South Korean population was divergent from east part of the Russian population even if it belonged to the lineage Far Eastern. Furthermore, we examined only maternal aspects here because of the limit of samples and data availability. Under the given circumstances, we proposed that the South Korean population of Siberian flying squirrel belongs to the lineage Far Eastern as one partial ESU of three partial ESUs but different management units. Future studies should consider the ecological surveys and other

genetic markers such as nuclear and Y chromosome genes as well as a wider sampling to figure out the partial and full ESU for the Siberian flying squirrel in South Korea.

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