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RESEARCH ARTICLE

MITOCHONDRIAL CYTOCHROME B GENE STUDY OF APODEMUS PENINSULAE IN MONGOLIA

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ABSTRACT

Korean field mouse (*Apodemus peninsulae*) are widely distributed throughout Northeastern Asia comprisingmany regions in many countries, including Russia, Japan, Korean Peninsulae, Mongolia and China. We analyzed mitochrondrial cytochrome b gene (1143 bp) of the *Apodemus peninsulae* and 11 specimens were collected from Bogd Khan Strictly Protected Area in Mongolia. We identified two different haplotypes from five new sequences. The genetic distance for Mongolian first haplotype was 0-1.8% while second haplotype depicts relatively high genetic distance (1.1-2.6%). This molecular genetic analysis demonstrates that *A.peninsulae* from the populations in regions of Russia such as Zabaikal, Khabarovsk, and Sakhalin are close to population in Mongolia in terms of the first haplotype. However the second haplotype from Mongolia is similar with the populations of Korea and China and form clade with strong maximium likelihood (86%) bootstrap support. As a result, we assume that there might be two different subspecies in Bogd Khan Strictly Protected Area of Mongolia.

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INTRODUCTION

Small mammals in Mongolia are interesting subject to study in terms of molecular phylogeny. The Korean field mouse, *Apodemus Peninsuale Thomas*, 1906 occurs across China, Korea, and northern Mongolia and extends to southern Siberia and the Russian Far East including the islands of Sakhalin and Hokkaido in Japan (Jones *et al*., 1956). Although molecular phylogeny and intra-species variation of this species was previously studied well using mitochondrial DNA markers (Serizawa *et al*., 2000 and 2002; Lui *et al*., 2004; Michaux *et al*., 2001; and Kim HR *et al*., 2011) in other countries such as Russia, Japan, Korea and China, data on genetic comparisons with *A.peninsulae* in Mongolia did not exist. The previous studies of this species exhibit variations in morphology, cytogenetics, biochemistry and genetics. Vorontsov *et al*., 1977 classified this species into six subspecies based on its morphology. The studies of B chromosomes and C band pattern of autosomes also depict difference among populations of Sakhalin and other part of Russia (Abe *et al*., 1997; Bekasova *et al*., 1975; Hayata 1973, Kral *et al*.,). While the populations of Sakhalin and Hokkaido played important role on cladogenesis, the populations from Primorye, Siberia and the Korean Peninsula can be the evidence for refugia for *Apodemus Peninsula* (Serizawa *et al*., 2000). Therefore three main clades were determined in the species, which are *A.peninsulae* distributed throughout East Siberia, Russian Far East, Korea and Japan (Serizawa *et al*., 2002). The phylogeny of genus *Apodemus* was studied based on nuclear and mitochondrial DNA markers by several scientists. The mice species were divided into four major groups including *Sylvaemus, Apodemus, Argenteus and Gurkha* (Serizawa *et al*., 2000) but based on the data of three genetic markers of nuclear IRBP, mitochondrial 12S rRNA and cytochrome b gene, 8 species of *Apodemus* group is sorted into two main groups: *Apodemus* (*A. agrarius*, semotus, and peninsulae) and a *Sylvaemus* group (with *uralensis, flavicollis, alpicola, sylvaticus*, and *hermonensis*). *Apodemus* group diverged at about 2.92-5.21 Mya (Liu *et al*., 2008). Even though the genetic analysis of this species was previously studied well by many scientists (Serizawa *et al*., 2000 and 2002; Lui *et al*., 2008; Michaux *et al*., 2001; and Kim HR *et al*., 2011; and Oh DJ *et al*., 2011), we are interested in knowing how Mongolian *A. peninsulae* is different from other populations. In the paper, we analyzed mitochondrial cytochrome b gene of *A. peninsulae* in Bogd Khan Mountain, Mongolia to compare with other populations in the world.

MATERIALS AND METHODS

The Korean field mouse (*Apodemus peninsulae*) samples were collected from 11 localities covering the species range in Bogd khan SPA, Ulaanbaatar. Much of the sample material consisted of meat tissue preserved in dried form or in absolute ethanol. Total genomic DNA was extracted using standard protocol (Sambrook *et al*., 1989). The entire cytochrome *b* gene (1140) of *A. peninsulae* was amplified with polymerase chain reaction

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(PCR), using primer sets (L14724 and H15149 and; the internal primers, L15162 and H15915) designed by Irwin *et al* (1991). All PCRs 10 µl of reaction mixture contained approximately 100ng of genomic DNA, 0.5 µM of each primer, 0.2 mM dNTPs, 1.5 mM MgCl, and 2.5 units of *Tag* DNA polymerase. PCR thermal cycle profiles were as follows: a pre-denaturing step of 94^0C for 5 min; denaturing step of 94^0C for 1 min, annealing at 50^0C for 1 min, and extension at 72^0 C for 2 min (35 cycles); plus a final extension at 72^0C for 10 min. The PCR products were stored at 4^0C. To remove primers and unincorporated nucleotides, amplified products were purified using QIAquick PCR purification kit (QIAGEN, USA). For sequencing, the purified PCR products were analyzed with an automated DNA sequencing machine (ABI 3770) in Korea. We used Network 4.6 version to identify the number of haplotypes from our new sequences. Published sequences for cytochrome b were retrieved from Genbank (Table 1) and aligned to our new sequences using the Codon code aligner and MEGA (version 5.05).

Table 1. Reference sequences of *Apodemus peninsulae* in Genbank (NCBI)

#	Place	Genbank accession number	Reference
1	Changchun, China	AY388999	Liu,X *et al.* 2004
2	Zabaikalys Siberia Russia	AB073803	Serizawa *et al.* 2001
3	Kabarovsk Russia	AF427338	Dekonenko *et al.* 2001
4	Japan	AB073788	Serizawa *et al.* 2001
5	Japan	AB073789	Serizawa *et al.* 2001
6	Japan	AB073790	Serizawa *et al.* 2001
7	Russia	AB073791	Serizawa *et al.* 2001
8	Sakhalin Russia	AB073792	Serizawa *et al.* 2001
9	Primorye Russia	AB073798	Serizawa *et al.* 2002
10	Shandong China	AB073801	Serizawa *et al.* 2002
11	Siberia	AB073802	Serizawa *et al.* 2002
12	Siberia	AB073804	Serizawa *et al.* 2002
13	Siberia	AB073805	Serizawa *et al.* 2002
14	Siberia	AB073806	Serizawa *et al.* 2002
15	Siberia	AB073807	Serizawa *et al.* 2002
16	South_Korea	AB073809	Serizawa *et al.* 2002
17	South_Korea	AB073810	Serizawa *et al.* 2002
18	Primorye Russia	AB073815	Serizawa *et al.* 2002
19	Primorye Russia	AB073816	Serizawa *et al.* 2002
20	Primorye Russia	AB073817	Serizawa *et al.* 2002
21	South Korea	AB073818	Serizawa *et al.* 2002
22	Japan	AB032850	Serizawa *et al.* 2000
23	Haili, China	AY389000	Liu,X *et al.* 2004
24	Korea	HQ660074	Oh,D.J *et al.* 2011
25	Korea	HM034941	Oh,D.-J *et al.* 2011
26	Sakhalin Russia	AB073793	Serizawa *et al.* 2002
27	Magadan Russia	AB073795	Serizawa *et al.* 2002
28	Milang Korea	AB073811	Serizawa *et al.* 2001
29	Mongolia	JQ664593.1	This study
30	Mongolia	JQ664594.1	This study
31	Mongolia	JQ664595.1	This study
32	Mongolia	JQ664596.1	This study
33	Mongolia	JQ664597.1	This study
34	Sichuan China	AY389001	Liu,X *et al.* 2004
35	Shandog China	AY389002	Liu,X *et al.* 2004
36	Ultai Siberia Russia	AB073818	Serizawa *et al.* 2002

New haplotypes of cytochrome b gene in our sequences were revealed using DNAsp program. The genetic distances between the populations across China, Korea and northern Mongolia extending to southern Siberia and the Russian Far East including the islands of Sakhalin and Hokkaido in Japan were calculated using Tajima-Nei distance method (Tajima-Nei, 1984). One haplotype from Mongolia was separated as a different group in the nucleotide distance calculations. Phylogenetic trees were constructed by maximum likelihood method with 1000 replications based on the Tamura-Nei model (Tamura *et al*., 1993) which uses MEGA (version 5.05). The percentage of trees in which the associated taxa clustered together is depicted next to the branches. The sequence of a house mouse, *mus musculus* (Genbank accession number BAA95807), was used as an out-group.

RESULTS

Complete sequences of cytochrome b gene of Korean field mouse (*A. peninsulae*) were sequenced. Five new sequences from 10 specimens in Bogd Khan Strictly Protected Area in Mongolia were identified and those sequences were submitted to Genbank (The accession numbers are JQ664593, JQ664594, JQ JQ664595, JQ JQ664596 and JQ664597). We identified two new haplotypes from the five new sequences using Network 4.6 version. New sequence with the accession number, JQ664597 was indicated as a haplotype individually while other four sequences were depicted as a different haplotype. Tamura-Nei distances among of *A. peninsulae* in Mongolia are given in Table 2 (haplotypes are labeled as in Fig. 1). Base composition of these sequences was skewed toward deficiencies in guanine (15.36%) and cytosine (19.44%). The other two nucleotides were more balanced (thymine 33.54%, and adenine 31.66%). However the base composition of the second haplotype (JQ664597) was slightly different (adenine 31.03%, cytosine 18.81%, guanine 15.99% and thymine 34.17%) compared with the other sequences. The genetic distances of the first Mongolian group among the other populations are between 0 and 1.9% while second group depicts a big genetic distance (1.1%-2.9%) compared with the other populations (Table 1).

To get a better understanding of evolutionary relationships between the populations, phylogenetic trees of 36 sequences of mitochondrial cytochrome b gene of *A. peninsulae* were constructed by Maximium likelihood method with 1000 bootstrapped replications. Maximum parsimony and neighbor joining trees are not shown, as they were essentially similar to neighbor joining tree (Fig.1). The entire population diversity is 0.0153 (S.E=0.0036). The distances were calculated from the sequence data. The numbers of base substitutions per site between sequences are depicted. Standard error estimate(s) are depicted above the diagonal. Analyses were conducted using the Tamura-Nei model (Tamura K *et al*., 1993) which applies MEGA (version 5.05). The molecular phylogenetic trees and the genetic distance results show that *A.peninsulae* from the Russian populations from regions such as Zabaikal, Khabarovsk, Sakhalin are close to Mongolian population. Korean population exhibit specific patterns in the cyt b gene sequences, which differ from those of Russian individuals from Primorye (Serizewa *et al*., 2002) and in same way Mongolian population for the first haplotype have an mtDNA lineage distinct from the Korean and Chinese populations of *A.peninsulae*. However the second haplotype from Mongolia is similar with the populations of Korea and China and forms clade with strong maximium likelihood (86%) bootstrap support (Figure 1). Optimal maximum likelihood tree for a dataset (L=-911) of 36 *Apodemus peninsulae* using 319 positions of the cytochrome b gene is depicted. *Mus musculus* is used as an out-group.

Table 2. Estimates of genetic distances between groups of *Apodemus peninsulae*

#	Place	1	2	3	4	5	6	7	8	9	10
1	China		0.005	0.005	0.004	0.005	0.005	0.005	0.004	0.005	0.006
2	Zabaikalys Russia	0.019		0.004	0.003	0.003	0.004	0.003	0.007	0.000	0.009
3	Kabarovsk Russia	0.020	0.006		0.003	0.003	0.004	0.004	0.007	0.004	0.009
4	Hokkaido Japan	0.017	0.003	0.003		0.002	0.003	0.003	0.006	0.003	0.008
5	Sakhalin Russia	0.018	0.005	0.005	0.002		0.003	0.004	0.006	0.003	0.008
6	Primorye Russia	0.023	0.010	0.009	0.007	0.009		0.004	0.007	0.004	0.009
7	Siberia Russia	0.022	0.010	0.013	0.010	0.012	0.015		0.005	0.003	0.007
8	Korea	0.018	0.018	0.017	0.015	0.016	0.021	0.017		0.007	0.005
9	Mongolia 1	0.019	0.000	0.006	0.003	0.005	0.010	0.010	0.018		0.009
10	Mongolia 2	0.024	0.026	0.026	0.022	0.024	0.029	0.022	0.011	0.026	

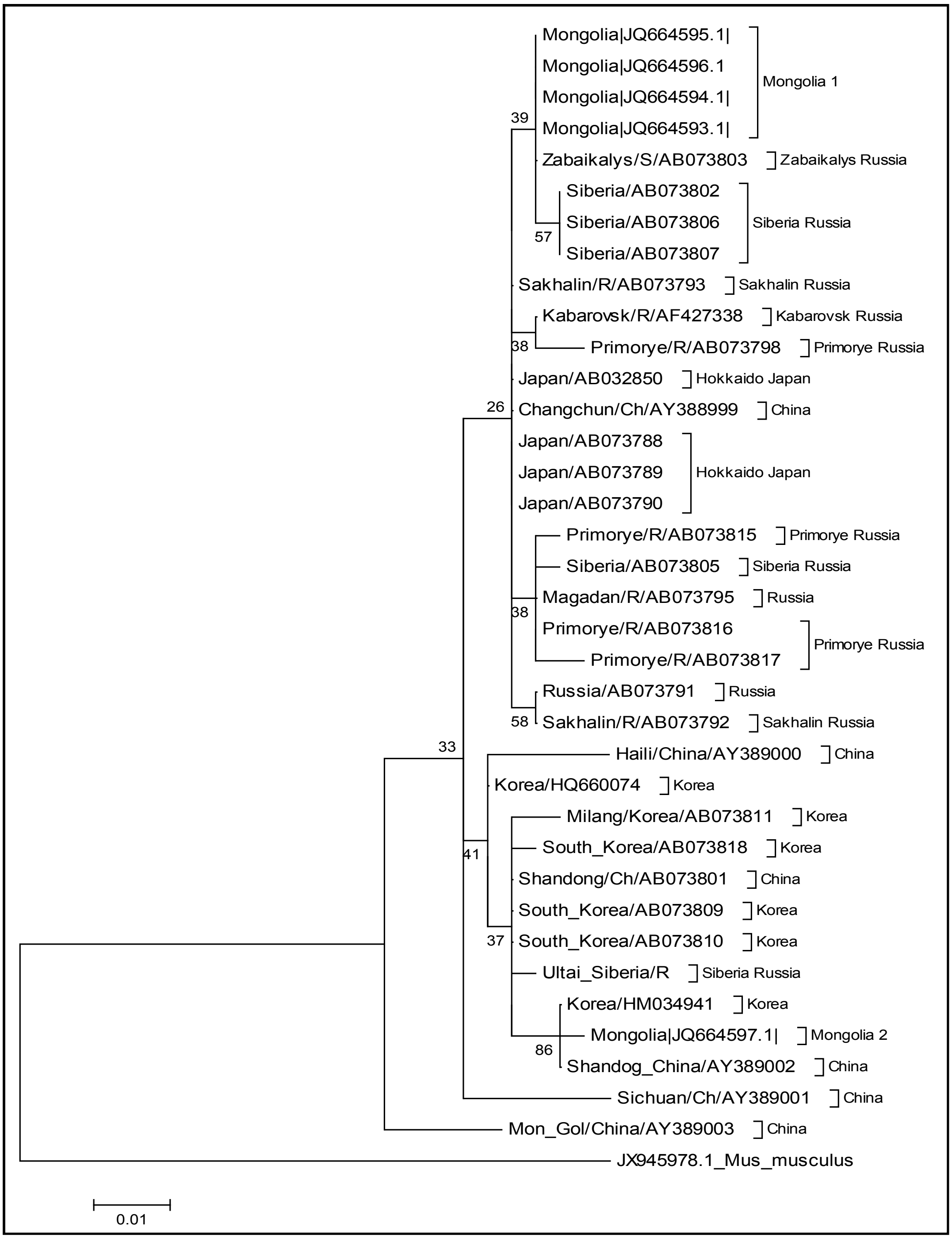

Figure 1. Phylogenetic tree of Korean field mouse (*Apodemus peninsulae*) with Mongolian popoluation. Phylogenetic trees were constructed by maximum likelihood method with 1000 replications based on the Tamura-Nei model (Tamura *et al.* 1993) using MEGA (version 5.05). The percentage of trees in which the associated taxa clustered together is depicted next to the branches. The sequence of a house mouse, *mus musculus* (Genbank accession number BAA95807), was used as an out-group.

DISCUSSION

The small mammals in Mongolia are much less studied genetically compared to other mammals. This species distributed in Northeastern Asia, which comprises many geographic regions such as Russian Primorye, Siberia, Khabarovsk, Sakhalin, Korean Peninsula, Japanese Hokkaido, Northern Mongolia and China where many mammalian species indicate different characteristics in morphology and genetics (Serizawa *et al*., 2000). It is interesting subject to study genetics of this species for understanding the influences of geographic distribution, history and ecology on formulation of population in Mongolia. We have analyzed the mitochondrial cytochrome b gene of *Apodemus Peninsulae* of Mongolia to compare with other populations in Russia, Korean Peninsula, China and Japan. Mitochondrial DNA is a valuable genetic marker for studies of evolutionary relationships of species (Irwin *et al*., 1991). Those 2 haplotypes were found from 5 new sequences in seven provinces, suggesting there might be high genetic variation in *Apodemus peninsulae* of Mongolia. First haplotype from Mongolia showed close genetic distances to Hokkaido Island in Japan, Russian populations from Zabaikal, Siberia, Sakhalin and Khabarovsk. However,an interesting observation was that second haplotype (JQ664597) was relatively different (1.1-2.9%) from others found in Mongolia (Table 2 and Figure 1). Considerable amounts of sequence divergences in the mitochondrial cytochrome b gene, as much as 2-4% can be observed among individuals from the areas of Northeastern Asia, implying that considerable time has passed since their ancestors colonized these geographic areas (Iwasa *et al*., 2000). Therefore the previous genetic studies of some mammals such as red squirrel and siberian roe deer in showed a slightly big genetic distance (Koh *et al*, 2013 and Bayarlkhagva *et al*., 2012). Hence, our result indicates that Mongolian populations can be denoted as refugia.

Based on genetic distances and phylogenetic trees, first haplotype from Mongolia has an mtDNA lineage distinct from the Korean and Chinese populations of *A.peninsulae* while the second haplotype is similar with the populations of Korea and China and forms clade with strong maximium likelihood (86%) bootstrap support (Figure 1). We assume that *A.peninsulae* from Mongolia highly diverged genetically in terms of the mtDNA cytochrome b gene. Recent cytochrome b gene study confirmed that Korean population, A.p. peninsulae is distinct from A.p.sowerbyi and A.p.preator from China (Hung Sun Koh *et al*., 2013). Accordingly we assume that there might be two subspecies of *Apodemus peninsulae* at Bogd khan Strictly Protected Area of Mongolia. Thus, further analyses of mtDNA cytochrome b gene with additional specimens of *Apodemus peninsulae* from Mongolia are needed to clarify the taxonomic status, reason of geographical affinity and environmental condition influencing the genetic variation in structuring the population.

Conclusion

Our study was concluded that Mongolian population of *Apodemus peninsulae* has a variety of genetic differences. Therefore there could be two individual subspecies of *A. Peninsulae*.

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