



MITOCHONDRIAL CYTOCHROME B GENE SEQUENCE DIVERSITY IN THE MONGOLIAN RED SQUIRREL, *Sciurus vulgaris* L.

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Received: February 02, 2012; Accepted: April 28, 2014

Abstract- Red squirrels (*Sciurus vulgaris*) are widely distributed throughout Eurasia, occurring in many types of coniferous and mixed-deciduous forests. Even though red squirrels are biologically and genetically well-studied worldwide, so far no genetic studies have been conducted in Mongolia. In this paper, complete sequences of the mitochondrial cytochrome b gene of the Mongolian red squirrel (*Sciurus vulgaris*) were analyzed to determine genetic diversity. Fifteen specimens were collected from seven provinces: Bulgan, Selenge, Tuv, Khuvsgul, Arkhangai, Zavkhan and Bayan-Ulgii. Ten haplotypes were observed from 15 specimens in seven Mongolian provinces, and the maximum Tamura-Nei nucleotide distance among them was 1.1%, indicating that genetic diversity of *Sciurus vulgaris* is moderate. The population in Khuvsgul showed the highest genetic distance compared to individuals the remaining populations in Mongolia. Further analyses of mtDNA cytochrome b gene with additional specimens of red squirrels from Khuvsgul province are needed to clarify the reason of environmental condition influencing the genetic variation in the squirrel's population in Khuvsgul.

Keywords- mitochondrial DNA, cytochrome b gene, red squirrel

Citation: Bayarkhagva D., Bayarmaa G. and Odbayar T. (2014) Mitochondrial Cytochrome B Gene Sequence Diversity in the Mongolian Red Squirrel, *Sciurus vulgaris* L. International Journal of Genetics, ISSN: 0975-2862 & E-ISSN: 0975-9158, Volume 6, Issue 1, pp.-149-152.

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Introduction

Sciurus vulgaris L. 1758 (Glires, Rodentia, Sciuridae), the Eurasian red squirrel, has the largest range of all tree squirrel species. It is distributed throughout the Palearctic forests from Siberia and Great Britain; east to Kamchatka Peninsula and Sakhalin of Russia and Hokkaido of Japan; south to the Mediterranean and Black Seas; and in northern Mongolia, and western and northeastern China [1,2]. Although red squirrels have been studied well in other areas [3,4], we are still far from having a clear understanding of the widespread and popular animal, squirrel *Sciurus vulgaris*, in Mongolia.

DNA sequences have become the most frequently used taxonomic characters to infer phylogenetic history [5], and mitochondrial DNA (mtDNA) is a highly sensitive genetic marker suitable for studies of closely related taxa or populations of a variety of species [6]. From the phylogenetic analyses of red squirrels in a recent study, two main mitochondrial phylogroups were revealed in Europe [7]. The first clade is from the region of Calabria in southern Italy, belonging to the subspecies *S.v.meridionalis*, while the second clade is from *S.v.infuscatatus* [8]. In this paper, we analyzed 15 sequences of mtDNA cytochrome b (*cyt b*) gene of the Mongolian red squirrel (*Sciurus vulgaris*) to determine the degree of genetic diversity. We found 10 new haplotypes of cytochrome b gene in the Mongolian red squirrel population, previously unreported in Genbank.

Materials and Methods

Red squirrel samples were collected from seven Mongolian provinces in which the species is currently found [Fig-1]. Fifteen of these specimens of *S. vulgaris* were sequenced. Muscle tissues were preserved in a deep freezer at -70°C. From muscle samples, total cellular DNA was extracted as follows: 500 µL STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA pH 8.0), 25 µL of 10 mg/mL stock of proteinase K, and 25 µL of 20% SDS were added into a microtube containing minced tissue. It was incubated at 55°C for 2h, and DNA was extracted with equal volumes of PCI and chloroform, and then was precipitated with 2 volumes of ethyl alcohol. After adding RNase A (10mg/ml), the solution was incubated at 37°C for 2h, and DNA was extracted again.

The entire cytochrome b gene (1140 bp) of red squirrels was amplified with polymerase chain reaction (PCR), using a primer set (L14724 5'-GATATGAAA AACCATCGTTG-3', and H15149 5'-GATTTTGG TTTACAAGACCGAG-3') designed by Irwin *et al.* [9]. The 50 µl of reaction mixture contained approximately 100 ng of genomic DNA, 25 pM of each primer, 200 µM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5mM MgCl₂, and 2.5 units of *Taq* DNA polymerase. PCR thermal cycle profiles were as follows: 94°C for 5 min; 94°C for 1 min, 55°C for 1 min, 72°C for 2 min (35 cycles); and 72°C for 10 min. To remove primers and unincorporated nucleotides, amplified products were purified using QIAquick PCR purification kit.

tion kit (QIAGEN, USA). For sequencing, the purified PCR products were analyzed with an automated DNA sequencer (ABI 3770) at Seoul National University, Korea.

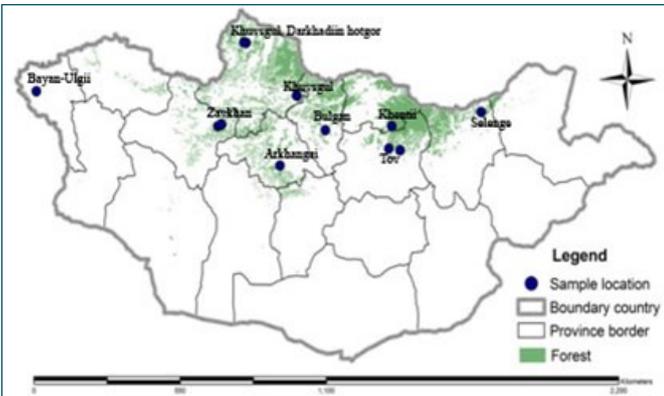


Fig. 1- Specimens' location of Mongolian Red squirrel, *Sciurus vulgaris*

Fifteen haplotypes in the complete mitochondrial cytochrome b (1140 bp) gene of the Mongolian red squirrel (*Sciurus vulgaris*) were aligned. The corresponding six sequences from Japan (Genbank accession numbers: AB292679, AB292680, AB292681, ABO30026, ABO30027 and ABO30028); and twenty-two sequences from Europe (Genbank accession numbers FJ932485, FJ932486, FJ932487, FJ932488, FJ932489, FJ932490, FJ932491, FJ932492, FJ932493, FJ932494, FJ932495, FJ932496, FJ932497, FJ932498, FJ932499, FJ932500, FJ932501, FJ932502, FJ932503, FJ932504, FJ932505, NC_002369; and AJ238588) were obtained from Genbank, and all 38 sequences were compared. Tamura-Nei distances [10] were calculated and phylogenetic trees were constrained by un-weighted Maximum Likelihood with 500 replications, Neighbor-joining with 2000 replications and Maximum Parsimony methods with 1000 bootstrapped replications and maximum parsimony methods using MEGA (version 5.05). The sequence of the eastern fox squirrel, *Sciurus niger* (Genbank accession number U10180.1), was used as an outgroup.

Results

Complete sequences of cytochrome b gene of squirrel *S.vulgaris* were sequenced. Ten new haplotypes from 15 specimens at Seven provinces in Mongolia were found: Mongolia 1UB (one from Ulaanbaatar), Mongolia 2Zav (three from Zavkhan), Mongolia 3Ar (one from Arkhangai), Mongolia 4Ba-UI (one from Bayan-Ulgii), Mongolia 5Zav (one from Zavkhan), Mongolia 6Tuv (one from Tuv), Mongolia 7Sel (one from Selenge), Mongolia 8Bul (one from Bulgan), Mongolia 9Khu (two from Khuvsgul), Mongolia 10Khu (one from Khuvsgul). These new haplotypes were submitted to Genbank. (The accession numbers, JQ395045, JQ395046, JQ395047, JQ395048, JQ395049, JQ395050, JQ395051, JQ395052, JQ395053 and JQ395054 were taken from Genbank).

Tamura-Nei distances among 10 haplotypes of squirrel *S. vulgaris* in Mongolia are given in [Table-1] (haplotypes are labeled as in [Fig -1]. Base composition of these sequences was skewed toward a deficiency in guanine (13.69%). The other three nucleotides were more balanced (thymine 33.21%, cytosine 26.99% and adenine 26.11%). Base frequency across in-group taxa was homogeneous ($\chi^2=6.32$, $df=42$, $P=1.0$). Maximum nucleotide distance among ten haplotypes of red squirrel in Mongolia was 1.1%. The haplotypes

from the squirrels in Khuvsgul (Darkhadiin hotgor) and Zavkhan have comparatively different haplotypes and more genetic divergence [Table-1], [Table-2] and [Fig-2].

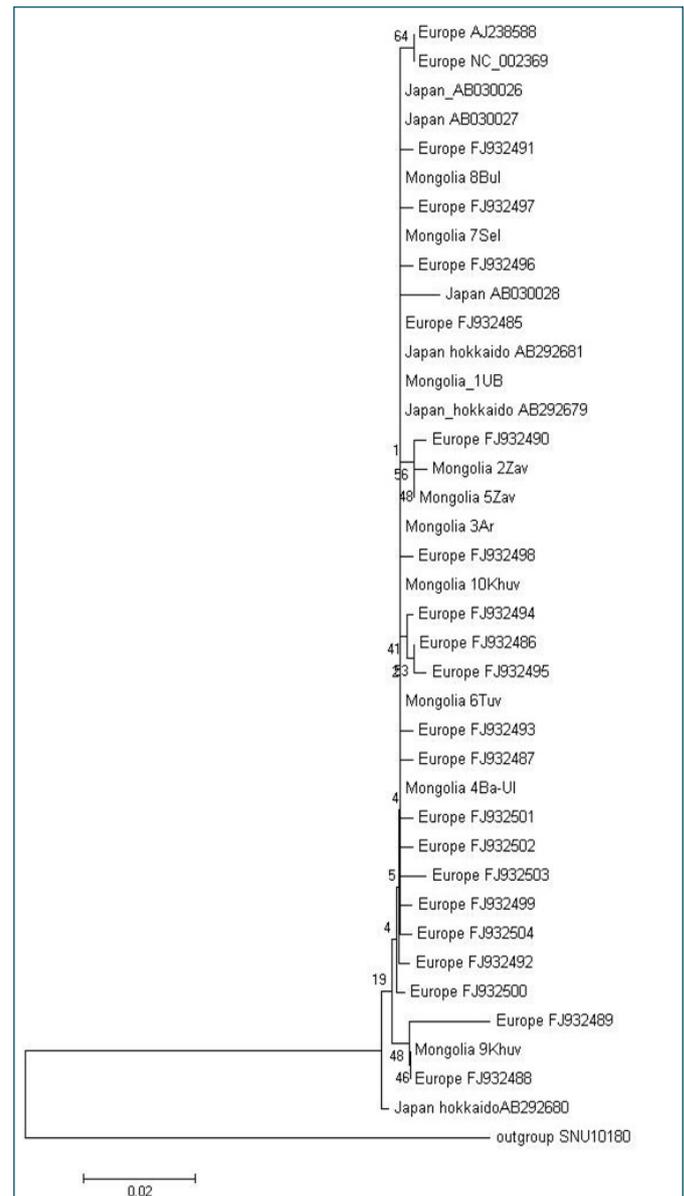


Fig. 2- Neighbor joining treewith 39 haplotypes of mtDNA cytochrome b of red squirrel, *Sciurus vulgaris*. The 10 haplotypes of Mongolia 1UB, Mongolia 2Zav, Mongolia 3Ar, Mongolia 4Ba-UI, Mongolia 5Zav, Mongolia 6Tuv, Mongolia 7Sel, Mongolia 8Bul, Mongolia 9Khu, and Mongolia 10Khu are from Mongolia. The corresponding six sequences from Japan (Genbank accession numbers: AB292679, AB292680, AB292681, ABO30026, ABO30027 and ABO30028); twenty two sequences from Europe (Genbank accession numbers FJ932485-FJ932505, NC_002369; and AJ238588) were obtained at this study. All 39 haplotypes were used to calculate Tamura-Nei nucleotide distances, and a neighbor joining tree with 2000 bootstrapped replications was constructed.

The phylogenetic trees of 38 haplotypes of mitochondrial cytochrome b gene of squirrel *S. vulgaris* were constructed by Neighbor joining with 2000 bootstrapped replications and maximum parsimony and maximum likelihood trees are not shown, as they were es-

entially similar to neighbor joining tree [Table-2] [11]. Analysis of this complete cytochrome b gene (1140bp) revealed 1.1% se-

quence divergence among all haplotypes from red squirrels throughout Mongolia.

Table 1- Estimates of Evolutionary Divergence between 10 haplotypes of Mongolian red squirrel

	B1UB	B2Zav	B3Ar	B4Ba-UI	B5Zav	B6Tuv	B7Sel	B8Bul	B9Khuv
B2Zav	0.004								
B3Ar	0.002	0.004							
B4Ba-UI	0.001	0.004	0.001						
B5Zav	0.004	0.001	0.004	0.003					
B6Tuv	0.002	0.004	0.002	0.001	0.004				
B7Sel	0.002	0.004	0.002	0.001	0.004	0.002			
B8Bul	0.001	0.004	0.001	0	0.003	0.001	0.001		
B9Khuv	0.01	0.011	0.01	0.009	0.01	0.01	0.01	0.009	
B10Khuv	0.001	0.005	0.003	0.002	0.004	0.003	0.003	0.002	0.011

The distances were calculated from the sequence data. The numbers of base substitutions per site between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model [12] using MEGA (version 5.05).

Table 2- Tamura-Nei distances among 16 Haplotypes of Cytochrome b Nucleotide Sequences (1140 bp) in *S. vulgaris* specimens

Haplotype	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	AB030026	AB292679	AJ238588	FJ932485	FJ932502
B1UB															
B2Zav	0.006														
B3Ar	0	0.006													
B4Ba-UI	0	0.006	0												
B5Zav	0.003	0.003	0.003	0.003											
B6Tuv	0	0.006	0	0	0.003										
B7Sel	0	0.006	0	0	0.003	0									
B8Bul	0	0.006	0	0	0.003	0	0								
B9Khuv	0.006	0.011	0.006	0.006	0.008	0.006	0.006	0.006							
B10Khuv	0	0.006	0	0	0.003	0	0	0	0.006						
AB030026	0	0.006	0	0	0.003	0	0	0	0.006	0					
AB292679	0	0.006	0	0	0.003	0	0	0	0.006	0	0				
AJ238588	0.003	0.008	0.003	0.003	0.006	0.003	0.003	0.003	0.008	0.003	0.003	0.003			
FJ932485	0	0.006	0	0	0.003	0	0	0	0.006	0	0	0	0.003		
FJ932502	0.003	0.008	0.003	0.003	0.006	0.003	0.003	0.003	0.008	0.003	0.003	0.003	0.006	0.003	
NC_002369	0.003	0.008	0.003	0.003	0.006	0.003	0.003	0.003	0.008	0.003	0.003	0.003	0	0.003	0.006

Table 3- Sequence differences (1140 bp) of mitochondrial DNA cytochrome b gene between 10 haplotypes in Mongolia

Position	52	67	69	150	306	364	402	498	501	513	570	627	702	786	873	907	960	1029	Total differences
consensus	T	G	T	C	T	G	A	C	A	T	G	T	T	T	C	T	A	T	
B1UB	T	G	T	C	T	G	A	C	A	T	G	T	C	T	C	T	A	T	1
B2Zav	T	G	T	C	T	A	G	C	A	T	A	T	T	T	C	C	A	T	4
B3Ar	T	G	T	C	T	G	A	C	A	T	G	T	T	T	T	T	A	T	1
B4Ba-UI	T	G	T	C	T	G	A	C	A	T	G	T	T	T	C	T	A	T	0
B5Zav	T	G	T	C	T	A	A	C	A	T	A	T	T	T	C	C	A	T	3
B6Tuv	T	G	T	C	T	G	A	C	A	T	G	T	T	T	C	T	A	C	1
B7Sel	T	A	T	C	T	G	A	C	A	T	G	T	T	T	C	T	A	T	1
B8Bul	T	G	T	C	T	G	A	C	A	T	G	T	T	T	C	T	A	T	0
B9Khu	A	G	C	T	C	G	A	T	G	C	A	C	T	C	C	T	A	T	10
B10Khu	T	G	T	C	T	G	A	C	A	T	G	T	C	T	C	T	G	T	2
Summary:	9T,1A	9G,1A	9T,1C	9C,1T	9T,1C	8G,2A	9A,1G	9C,1T	9A,1G	9T,1C	7G,3A	9T,1C	8T,2C	9T,1C	9C,1T	8T,2C	9A,1G	9T,1C	23

Discussion

This study has quantified mitochondrial cytochrome b gene diversity in red squirrels in Mongolia. The red squirrel of Mongolia is much less studied biologically and ecologically compared to other mammals in Mongolia. Included in the IUCN red list category of threatened species, the Mongolian red squirrel is near threatened/ least concern [13].

That 10 haplotypes were found from 15 specimens in seven provinces, suggesting there might be high genetic variation in red squirrels of Mongolia. An interesting observation was that Khuvsgul

(B9Khu) and Zavkhan (B2Zav and B5Zav) provinces' haplotypes were quite different from others found in Mongolia. The specimen B9Khu is from Darkhadiin hotgor, an area that is ecologically and climatically similar to Siberia. Further analyses of mtDNA cytochrome b gene with additional specimens of red squirrels from Khuvsgul province are needed to clarify the reason of environmental condition influencing the genetic variation in the squirrel's population in Khuvsgul.

Based on genetic distances and phylogenetic trees, there was generally a similar level of sequence divergence among haplotypes

within populations of Selenge, Bulgan, Tuv, Ulaanbaatar, Arkhangai and Bayan-Ulgii. Mitochondrial DNA is a valuable genetic marker for studies of evolutionary relationships of species [9]. Independent sources of characters, such as nuclear and mtDNA, reflect different but equally accurate phylogenetic patterns [14]. Conservative patterns of protein differentiation previously reported for *Tamiassibiricus*' subspecies now appear to be paralleled by a conservative pattern of mtDNA divergence; it seems some mammal's mitochondrial DNA is more conservative related to the same environmental conditions [15, 16].

Generally, the mtDNA coding region genes evolve slower than non-coding region sequences [17]. Thus, cytochrome b gene variation has also diverged slowly in red squirrel of Mongolia.

Acknowledgement: The project research team would like to express their deepest gratitude to Asia Research Center at the National University of Mongolia, and Foundation for Advanced Studies of Korea for which they have supported and funded the "Research on sequencing of Cytochrome b (Cyt-b) gene of Mitochondrial DNA (mtDNA)" project implemented in Department of Genetics and Molecular Biology, School of Biology and Biotechnology, National University of Mongolia.

Conflicts of Interest: None declared.

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