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RESEARCH ARTICLE

PHYLOGENETICS RELATIONSHIP OF WILD BOARS (*SUS SCROFA* L. 1758) IN MONGOLIA

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ABSTRACT

Euroasian wild boar (*Sus scrofa*) is widely distributed throughout Southern Europe, Asia, and North Africa in Scrub, forest, and arid environments. Even though wild boars are biologically and genetically well-studied worldwide, so far no genetic studies have been conducted in Mongolia. Two subspecies of wild boars including *Sus scrofa nigripis* and *Sus scrofa raddeanus* are known in Mongolia based on their morphological differences. Then we needed to test genetic differentiation between those subspecies and compare geographically close populations in Mongolia using mitochondrial DNA complete *cytochrome b* gene. We also retrieved sequences of wild boars in the neighboring countries from Genbank to do phylogenetic relationships. Wild boars' tissue samples were collected from ten provinces throughout the distribution regions of wild boars in Mongolia. In result of the research, *Sus scrofa nigripis* showed a significant genetic difference as a subspecies. In conclusion, this research proven that there are two subspecies of wild boars in Mongolia according to the mtDNA data. Although our data can be enough to provide that *Sus scrofa nigripis* fits with the criteria of Evolutionary Significant Unit (ESU), in future big sample size based on non-invasive sampling method from those populations will be useful to identify the further questions.

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INTRODUCTION

Wild boar has the widest natural range of any ungulate or hoofed mammal, in the world. Outside of its natural range the wild boar has been introduced to many other parts of the world. The wild boar (*Sus scrofa*) was classified as four distinct subspecies in Europe, West Asia and Northwest Asia based on their morphological differences and geographical references (Genow, 1999; Groves ,1993). However, the genetic studies already resolved its taxonomy and classified more subspecies in Europe and Asia (Randi, 1995; Frantz, 2013; Watanobe, 1999). Although the most of the wild boar populations are studied well in Europe and Asia, the Mongolian wild boar populations are not well known genetically. Based on morphological data, two subspecies, *Sus scrofa nigripes* and *Sus scrofa raddeanus* (*Sus scrofa sibiricus*) are distributed in Mongolia (Shiirevdamba, 2013).

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Sus scrofa nigripes inhabits the forested regions of western Mongolia, including Great Lakes Depression and western Mongol Altai Mountain Range, while *Sus scrofa raddeanus* occurs in eastern parts of the country, including Khangai, Khuvsgul and Khentii mountain ranges, Ikh Khyangan Mountain Range and Mongol Daguur Steppe. In Mongolia, no data on population sizes are available at present, although it is known that threats, particularly exploitation is having a large impact upon this species, coupled with hybridization and habitat degradation (Dulamtseren, 2006). The wild boar fulfills an essential role in the ecosystem, because of its large size and frequent rooting for food. Generation length has been estimated as four years, based on data from (Nowak, 1991). As exploitation is known to be causing a population decline, upon the availability of the data, this species may be re-categorized as threatened under Criterion A. There is a small chance of immigration from adjacent populations of *S. s. sibirica*, although levels of hunting pressure on these populations are not known, therefore the assessment remains unchanged following application of regional criteria (Dulamtseren, 2006). Mitochondrial DNA (mtDNA) has been one of the most widely used molecular markers for phylogenetic studies in

animals, because of its simple genomic structure (Kocher, 1989). Although mitochondrial DNA is only shows the maternal lineage far back in time, mtDNA is a highly sensitive for phylogenetic studies of closely related taxa or populations of a variety of species.

MATERIALS AND METHODS

Total 18 wild boars' tissue samples were collected from ten provinces in Mongolia (Fig. 1 and Attachment 1).

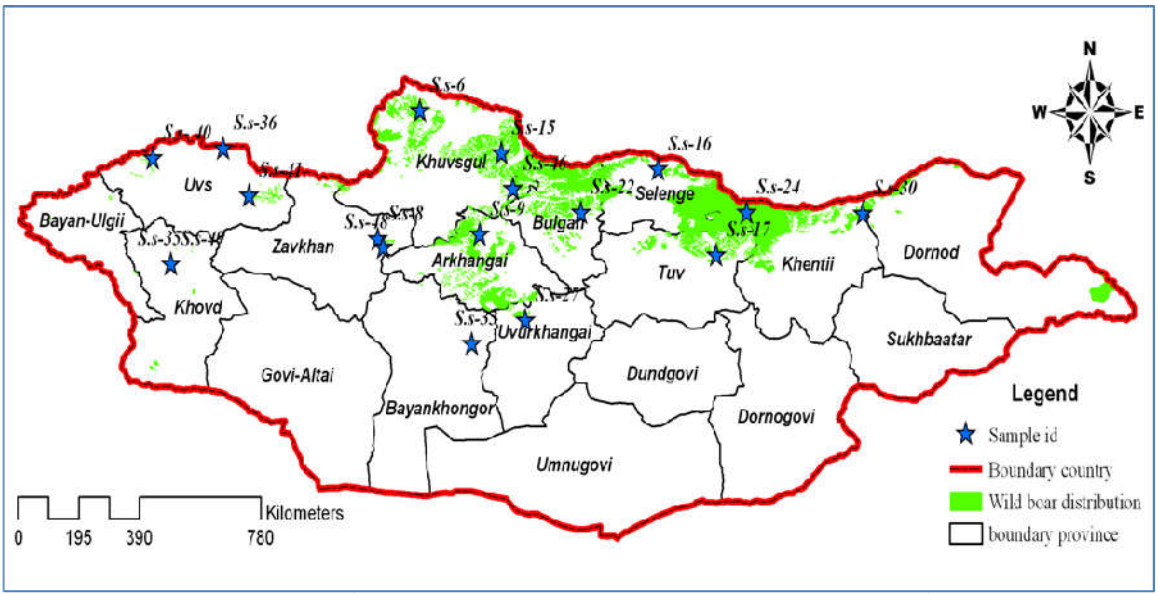

Figure 1. The Mongolian wild boar approximate distributions have been used from the from Mongolian Red book (Shiirevdamba, 2013) and IUCN Red List report (Oliver, 2014)

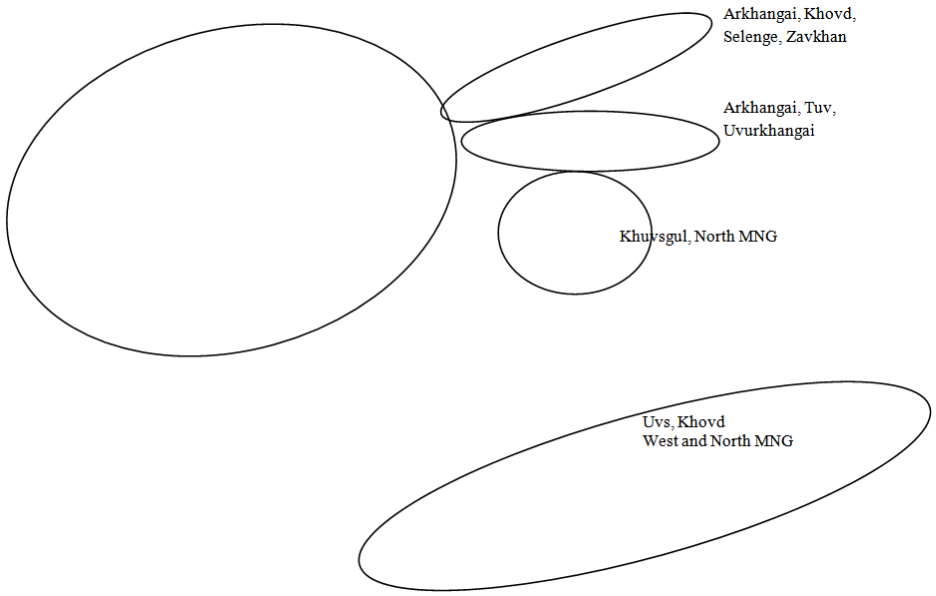

Figure 2. Network analysis

The samples were preserved at -20°C. Total genomic DNA was extracted as follows: tissue samples were grinded in liquid nitrogen and lysed in a buffer (500 µL STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA pH 8.0), 25 µL of 10 mg/mL of proteinase K, and 25 µL of 20% SDS) at 55°, and DNA was extracted with equal volumes of phenol:chloroform:isoamylalcohol, and then was precipitated with 2.5 volumes of 96% cold ethanol and 3M acetate Na. The entire *cytochrome b* gene was amplified with polymerase chain reaction (PCR) using primers *Cyt b* F:5'-CAC GAC CAA TGA CAT GAA AAA TC-3' and *Cyt b* R:5'-TGG CCC TCC TTT TCT GGT TTA C-3'. The 20 µl of PCR reaction mix contained approximately 100 ng of genomic DNA, 10 µM of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, and 1.0 units of *i*Taq DNA polymerase (Intron Biotechnology, Korea). PCR conditions were as follows: initial denaturation at 94°C for 2 min, 25 cycles of 94°C for 30 sec; 55°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min. PCR products are sequenced with the PCR primer pair (Invitrogen)

and BigDye terminator v.2 cycle sequencing kit (Applied Biosystems) on ABI Prism 310 Genetic Analyzer (Applied Biosystems) according to the manufacturers' specifications.

Data analysis

The sequences are edited using *Geneious* version 8.0.3 (Drummond *et al*., 2011) using with further modification by eye and *Codon Code aligner* (www.codoncode.com). In total 20 DNA sequences from other geographical regions such as eastern and central China, eastern Russia, Korea, Japan, Tibet and western Europe were retrived from NCBI genbank.

(http://ncbi.nlm.nih.gov/ NCBI accession numbers: AY237534; AF136547; AB015081; AF136549; AB015070; AM492581; DQ315603; AY634186; GU135689; GU135707; GU1357803; GU135820; JN601075; EF545584; HM010471; AB015065; KC505406; KC493612) and red deer (*Cervus elaphus*) was used as an outgroup and compared the sequences of this study.

RESULTS

Complete sequences of *cytochrome b* gene (1140 bp) of wild boar (*Sus scrofa* L.) were determined. Base composition of these sequences is guanine 13.69%; thymine 33.21%; cytosine 26.99%; and adenine 26.11% within Mongolian wild boar population. The number of variable sites within Mongolian wild boar population was 50. Wild boars population of north western Mongolia (*Sus scrofa nigripes)* have 16 nucleotide polymorphic sites including 9 transitions and 7 transversions comparing with other populations (Table 2). Published 16 sequences of wild boar mtDNA cytochrome b gene from Genbank and 18 nucleotide sequences of Mongolian wild boar samples were used for the phylogenetic analysis. We found totally 11 haplotypes out of 18 sequences of cytochrome b gene from Mongolia wild boar populations. Haplotype 1 (as shown H_1) has three individuals from Arkhangai, Tov and Uvurkhangai provinces. H20 is from Selenge which differ by only one nucleotide from H1.

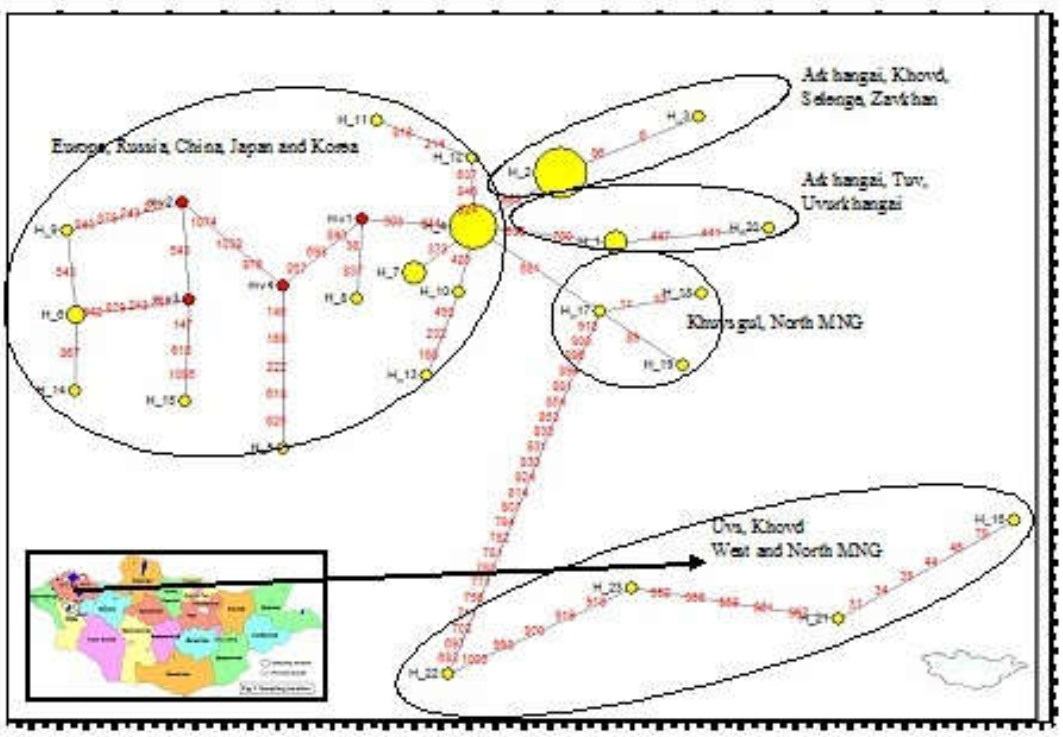

Figure 2. Haplotypes relationships are shown using MJ (Median joining) network algorithm (Hans-Ju¨rgen Bandelt and Arne Ro¨hl , 1999)

DNAsp 5 version (Rozas *et al.,* 2003) was used to identify the number of haplotypes from the new sequences and calculate genetic diversity among populations for mtDNA data. The network 4.6 version was used to see the relationship of the haplotypes. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 6 (Tamura *et al*., 2013). Phylogenetic trees were constructed with MEGA 6 using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980) with 1000 replications. Neighbor joining tree were also made, but it was similar with the result with the Maximum Likelihood tree. The pairwise FST values between populations are calculated using *Arlequin* 3.1 (Excoffier, 2010).

H2 (H_2) is the most common haplotype that occurs at the provinces of Arkhangai, Khovd-Jargalant, Selenge, Zavkhan. Therefore H2 occupied in the Chinese wild boar populations as well. H3 is from Dornod province at the eastern Mongolia. Although H4 differs from H2 by only one nucleotide (883th nucleotide) substitutions but we observed the haplotype from many places such as China, Taiwan, Vietnam, Japan, Russia, and Italy. Between all H5 and H15 are from Europe, China and Tibet but not found in Mongolia. H17, H18 and H19 are from Khuvsgul in North Mongolia and differ by 1-3 nucleotides substitutions. H21, H22 and H23 differ by many nucleotides substitutions (23-28 variable sites) from North and Western Mongolia.

Table 2 describtion: The molecular diversity index is calculated in *Arlequin* 3.1 (Excoffier, 2010). Although transversion mutation is higher in the population in North Mongolia (Khuvsgul), the mean ratio of transitions and transversions mutations is equal in within Mongolian wild boar populations. The number of polymorphic sites is slightly high in the wild boar population North Western Mongolia. The genetic distance is calculated by two different methods. The genetic distances on Table 1 is based on Kimura 2-parameter model (Kimura.M, 1980), table 2 is based on FST statistics using *Arlequin* 3.1 (Excoffier, 2010).

Table 1 description: The pairwise genetics distances were statistically significant. Analyses were conducted based the Kimura 2-parameter model (Kimura.M, 1980) using MEGA6 (Tamura K., 2013). The analysis involved 36 nucleotide sequences. The wild boar population in North Western Mongolia showed 2.9-4% of genetic distance but pairwise genetic distances between other wild boar populations in Euroasia are a slight low (0.1-1.5%) based on complete *cytochrome b* gene. Phylogenetic tree was constructed using MEGA6 (Tamura K., 2013) based on Kimura two parameter method (Kimura.M, 1980) with 1000 repetitions. Outgroup was used as red deer (*Cervus elaphus*).

Table 1. Distribution and frequency of haplotypes per location and domestic breed

#	Locations/No of Haplotypes	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	Samples
1	Uvs									1	1	1	3
2	Khuvsgul						1	1					2
3	Khovd		1		1	1							3
4	Arkhangai	1	1										2
5	Zavkhan		2										2
6	Selenge		2										2
7	Uvurkhangai	1											1
8	Orkhon								1				1
9	Tuv	1											1
10	Dornod			1									1
samples		3	6	1	1	1	1	1	1	1	1	1	18

Table 2. Molecular diversity index in Mongolian wild boar populations

Statistics	Mongolia Central provinces	North Western Mongolia	North Mongolia	Mean	s.d
No of transitions	4	9	0	3.250	3.700
No of transversions	3	7	3	3.250	2.487
Total substitutions	7	16	3	6.500	6.021

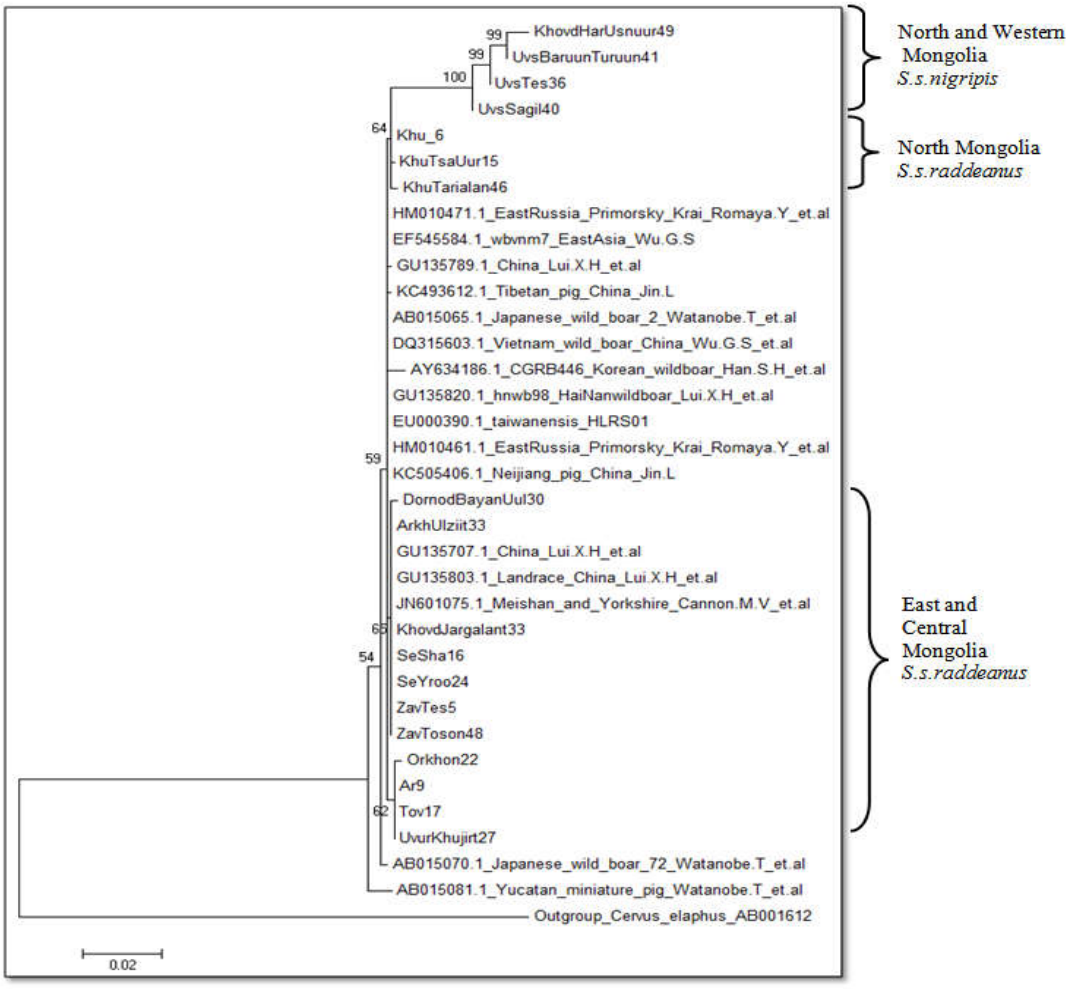

Figure 3. Wild boar (Sus scrofa) cytochrome b gene phylogenetic tree

Table 3. Pairwise genetic distance among populations based on mtDNA complete cytochorome b gene

	1	2	3	4	5	6	7	8	9
1.North West Mongolia									
2. North Mongolia	0.029								
3. Central Mongolia	0.031	0.004							
4.Central and East Mongolia	0.030	0.003	0.003						
5. China	0.029	0.002	0.003	0.001					
6. Russia	0.029	0.002	0.002	0.001	0.001				
7. Tibet	0.030	0.003	0.003	0.002	0.001	0.001			
8. Korea	0.033	0.006	0.007	0.006	0.005	0.004	0.005		
9. Japan	0.030	0.004	0.004	0.003	0.002	0.002	0.003	0.006	
10. Europe	0.040	0.013	0.013	0.012	0.012	0.011	0.012	0.015	0.011

Table 4. Population pairwise FSTs

	1	2	3
1. Central Mongolia			
2. Mongolia	0.60068*		
3. North Western Mongolia	0.84499*	0.88057*	
4. North Mongolia	0.75000	0.49629*	0.80992*

*- values are statistically significant

DISCUSSION

Mongolia is a wide country that some mammals are distributed in different ecological and isolated in geographically. So it is important to determine the gene flow between populations and study the phylogenetic relationships of the isolated small populations of wild animals for their future conservation management. Based on the morphological and ecological differences, wild boars in Western Mongolia consider as a different subspecies, *Sus scrofa nigripis* (Shiirevdamba, 2013). Therefore wild boar included in the IUCN red list category of least concern species (Oliver, 2014), the Mongolian wild boar is near threatened and can be classified in the threatened species due to illegal hunting and livestock pressure. Central Mongolian populations also share a haplotype (H2) of *cytochrome b* gene with the wild boar populations in China. Larson *et al*., 2005 also found that central Chinese wild boar populations share a few haplotypes of mtDNA control region with the southern Asian populations. Some authors (Fang M, 2006; Scandura, 2008) also found that asian mtDNA haplotypes were also found low frequency in some European wild boar populations. However, the absence of Mongolian wild boar haplotypes in the European wild boar populations (H5-H15) can be indicative of a limited historical gene flow.

Sequencing analysis of mitochondrial DNA *cytochrome b* gene revealed comparatively high level of gene diversity in wild boar populations in Mongolia. The genetic pairwise distances (Kimura.M, 1980) between the wild boar populations in western Mongolia and other wild boar populations in Mongolia, South Asia and Europe showed a significant differentiation (2.9-4%) while pairwise genetic distances between the populations was a slight low as 0.1-1.5%. The MJ network also clearly shows that north western Mongolian population is differentiated from Central and North Mongolian populations. Another interesting thing is that the haplotypes of Khuvsgul is differentiated by 1-3 nuleotides but do not share with other populations in Mongolia which is indicating that that there is a low genetic flow.

Therefore phylogenetic tree also shows that western Mongolian wild boar population can be a different clade. The present study provides new insight into the genetic diversity of Mongolian wild boar populations and their relationships with other Euroasian wild boar populations. Thus, western Mongolian population, *Sus scrofa nigripis* is genetically isolated which is supporting the hypothesis of different subspecies. The present should be expanded to nuclear markers and to the Y chromosome with more sampling from each population in Mongolia.

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