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ARTICLE

PHYLOGENETIC ANALYSIS OF MITOCHONDRIAL 12S RIBOSOMAL RNA GENE SEQUENCE OF MONGOLIAN WILD BOARS

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Abstract: *Specimens of Mongolian wild boar (Sus scrofa) from Arkhangai, Dornod, Zavkhan, Orkhon, Ovorkhangai, Selenge, Tuv, Khovd, Khuvsgul and Uvs aimags (provinces) were subjected to DNA sequencing. Determined sequences from 18 specimens were registered into the GenBank and accession numbers were obtained. In this study mitochondrial 12S rRNA gene sequences of Mongolian wild boars were analyzed with 36 complete sequences of 12S rRNA gene of wild boar (Sus scrofa) available at NCBI GenBank. Sequence alignment, detection of parsimonious informative sites, model selection, calculation of nucleotide distances and tree construction with 1000 bootstrapped replications were conducted using MEGA 6. Maximum likelihood trees were constructed by the HKY model. A maximum likelihood tree with 53 complete sequences of mitochondrial 12S rRNA gene of Sus scrofa was constructed. Mongolian sequences from the same and adjacent locations were clustered together.*
European sequences were clustered together, additionally two sequences from south western China and two sequences from south eastern China were also clustered. Additionally, 12S rRNA gene sequences of Mongolian Sus scrofa, located between Asian and European sequences suggesting geographical location of Mongolia, played an important role in the gene flow between Asian and European wild boar population.

Keywords: *Sus scrofa; mitochondrial genes; phylogenetic tree;*

INTRODUCTION

The use of molecular markers for species identification has become a powerful tool. In recent years, the use of DNA has been popularized because of its specificity and stability. Among types of DNA, mitochondrial DNA (mtDNA) has been used extensively as it has a high copy of mitochondria in cell. Mitochondrial DNA follows clonal inheritance as only mother contributes to mitochondrial DNA and its mitochondria does not undergo recombination, thus genetic material will be passed to the next generation unchanged. Studies have shown that mitochondrial genome accumulates high percentage of

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neutral mutations, which is helpful in species identification [11].

Mitochondrial genes are highly conserved in different species of animals which enable the designing of universal primers for their amplification [9]. Different mitochondrial genes have been used in species differentiation and identification [11]. Target genes and DNA fragments, used as markers, include cytochrome b gene, 16S and 12S rRNA gene and mitochondrial DNA control region [9]. Numerous molecular techniques have been developed based on the use of mitochondrial 12S rRNA gene, such as DNA hybridization [1], restriction fragment length polymorphism [6], real time PCR [5] and so forth.

The Eurasian wild pig has one of the widest geographical distribution of all hoofed mammals in the world, moreover, human activity played a major role in the expansion of its range. Based on both morphological and geographical differences, wild boar has four distinct subspecies groupings [3]. Based on morphological data, two subspecies, *Sus scrofa raddianus* and *Sus scrofa nigripes*, are distributed in Mongolia. *Sus scrofa nigripes* inhabits the forested regions of western Mongolia, including the Great Lake Depression and the western Mongolian Altai Mountain Ranges, while *Sus scrofa raddianus* occurs in eastern and northern parts of Mongolia, including Khangai, Khuvsgul and Khentii mountain ranges, Ikh Khyangan Mountain ranges and the Mongol Daguur Steppe [7].

Although wild boar populations are abundant and categorized as least concern in the world, in Mongolia no data on population of wild boars are available at present. However, it is known that threats, particularly exploitation, hybridization and environmental degradation are having huge impact on population size of wild boars in Mongolia [7]. Therefore, regionally, *Sus scrofa nigripes* may be re-categorized as threatened and *Sus scrofa raddianus* as near-threatened. There is a small chance of immigration from adjacent population of *Sus scrofa sibirica*, however, hunting pressure on these populations is unknown. Therefore assessment remains unchanged [7,8]. Moreover, wild boars are susceptible to a variety of highly contagious diseases which can decimate their population [9].

MATERIALS AND METHODS

Small pieces of tissue samples were collected and brought to laboratory under cold conditions or in ethanol. Samples were stored at -20°C until further processing. Tissue samples were ground in liquid nitrogen and lysed in 500 µl SNET (20 mM Tris-Cl pH 8.0, 5 mM EDTA pH 8.0, 400 mM NaCl, 1% SDS), 25 µl of 20 mg/ml of proteinase K and shaking vigorously at 55°C for 2-3 hours. DNA was extracted with equal volume of phenol: chloroform:isoamyl alcohol and precipitated with 2.5 volumes of 96% cold ethanol and 0.1 volume of 3M acetate Na. The integrity of isolated DNA was checked on 1.2% agarose gel electrophoresis and the purity was determined on the basis of optical density ratio at 260:280 nm.

Mitochondrial 12S rRNA gene was PCR-amplified using forward primer: 5’-GCCTAGATGAGCCTCACAGCT-3’ and reverse primer: 5’-ACATTGTGGGATCTTCTAGGTG-3’. Reaction was set up in 0.2 ml PCR tubes containing 10X buffer 2 µl, 200 mM dNTPs, 1 µ M of each primer, 200 ng template, 2.5 U enzyme (Takara) and nuclease free water to make the volume up to 20 µl.

The cycling conditions were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min. Final extension was carried out at 72°C for 10 min.

To confirm and check PCR products 1.2% agarose electrophoresis was performed and

subjected to DNA sequencing. Determined sequences were registered to GenBank and accession numbers were obtained.

Mitochondrial 12S rRNA gene sequences and accession numbers from Mongolian *S.scrofa* are given in Table 1. Complete mitochondrial 12S rRNA gene sequences were obtained from 18 specimens at 10 locations in Mongolia, as shown in Figure 1. These sequences were analyzed with complete sequences of mitochondrial 12S rRNA gene of *S.scrofa* (Table 2) available at NCBI GenBank.

Table 1. Location, coordinates, sequence name and accession numbers of Mongolian S.scrofa 12S rRNA gene used in this study

Sequence name	Location	Coordinates	GenBank accession number
	Mongolia		
MN1	Khuvsgul province	N51°25’32.15” E99°18’52.84”	KM520132
MN2	Zavkhan province	N48°28’20.86” E98°05’57.36”	KM520133
MN3	Arkhangai province	N48°35’13.33” E101°02’35.53”	KM520134
MN4	Tuv province	N47°43’23.84” E107°47’48.85”	KM520137
MN5	Khuvsgul province	N50°26’53.51” E101°40’33.27”	KM520135
MN6	Selenge province	N50°04’54.88” E106°11’44.43”	KM520136
MN7	Orkhon province	N49°05’02.66” E103°58’02.27”	KM520138
MN8	Uvs province	N49°22’15.21” E94°10’492.94”	KM520139
MN9	Uvurkhangai province	N46°35’57.48” E102°21’30.17”	KM520140
MN10	Dornod province	N49°03’41.51” E112°07’17.92”	KM520141
MN11	Arkhangai province	N46°03’53.06” E100°49’01.43”	KM520142
MN12	Khovd province	N47°54’17.36” E92°06’59.67”	KM520143
MN13	Uvs province	N50°33’53.22” E93°38’11.20”	KM520144
MN14	Uvs province	N50°20’12.16” E91°35’49.38”	KM520145
MN15	Uvs province	N49°28’27.01” E94°22’41.74”	KM520146
MN16	Khuvsgul province	N49°37’48.68” E102°00’34.85”	KM520147
MN17	Zavkhan province	N48°17’21.56” E98°15’16.15”	KM520148
MN18	Khovd province	N47°54’17.36” E92°06’59.67”	KM520149

Figure 1. Collection sites of 18 Sus scrofas pecimens from 10 locations in Mongolia. Three specimens (MN1, MN5, MN16) from Khuvsgul aimag, two (MN2, MN17) from Zavkhan, two (MN3, MN11) from Arkhangai, one (MN4 and MN6) from Tuv and Selenge aimags respectively, four (MN8, MN13, MN14, MN15) from Uvs, one (MN7) from Orkhon, one (MN9 and MN10) from Uvurkhangai and Dornod respectively and two (MN12, MN18) from Khovd aimag.

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Table 2. Location and accession numbers of S.scrofa obtained from GenBank. Eight complete sequences of mitochondrial 12S rRNA gene from Europe (one from Italy and Sweden, six from Spain), 27 complete sequences from Asia (seven from Korea, one from Vietnam and northern China, five from SE China, 11 from SW China, two from southern China) were used for this study.

Region	Location	GenBank accession number	Sequence name
Europe	Italy	AF304201	ITA1
	Sweden	AF304203	SWE1
	Spain	FJ236998, FJ236999, FJ237000 FJ237001, FJ237002, FJ237003	ESP1, ESP2, ESP3, ESP4, ESP5, ESP6
Asia	Korea	AY574047, DQ207753, DQ207754, DQ207755, DQ268530, EU090702, EU090703	KOR1, KOR2, KOR3, KOR4, KOR5, KOR6, KOR7
	China (South Eastern)	EF545579, EF545580, EF545569 EF545570, EF545571	CN1, CN2, CN3, CN4, CN5
	China (North Eastern)	EU333163	CN6
	China (South Western)	KC493607, KC493608, KC493609, KC493610, KC493611, KC493612 KC505411, EF545585, EF545586, EF545568, EF545573	CN7, CN8, CN9, CN10, CN11, CN12 CN13, CN14, CN15, CN16, CN17
	China (Southern)	KP681245, EF545572	CN18, CN19
	Vietnam	EF545584	VIE1

Complete sequences of mitochondrial 12S rRNA gene were obtained from 18 specimens of *Sus scrofa* at ten locations in Mongolia, as listed in Table 1. These sequences were compared to 36 complete sequences of mitochondrial 12S rRNA gene of *Sus scrofa* from four locations in Europe and eight locations in Asia, obtained from GenBank, as given in Table 2.

Sequence alignment, detection of parsimonious informative sites, model selection, calculation of nucleotide distances and tree construction with 1000 bootstrapped replications were conducted using MEGA 6[12]. The Hasegawa-Kishino-Yano (HKY) model [4], which showed the lowest Bayesian information criterion score was chosen as the best model for our data by the programme. Maximum likelihood trees were constructed by the HKY model. We decided to choose out-group not closely or distantly related to *Sus scrofa*. *Camelus bactrianus* (AP003423) was ideal candidate for out-group because it was in the same order as *Sus scrofa*.

RESULTS AND DISCUSSION

A maximum likelihood tree with 53 complete sequences of mitochondrial 12S rRNA gene of *Sus scrofa* is shown in Figure 2. The 53 sequences from nine regions in Eurasia (Gp1 – Mongolia; Gp2 – Korea; Gp3 – Italy, Sweden and Spain; Gp4 – south eastern China; Gp5 – north eastern China; Gp6 – south western China; Gp7 – southern China; Gp8 – Vietnam) were clustered according to their respective locations and bootstrap value supporting all sequences was 25. Average distance among populations (Table 3) in Asia (Gp2 and Gp4 to 8) ranged from 0% to 0.9% and in Europe (Gp3) was 0.1%, average distance of Mongolian wild boar populations was 0.6%. On the other hand, average distance between populations (Table 4) ranged from 0% to 1%. Average distance between Mongolian and other populations ranged from 0.3% to 0.8%. Highest average distance between populations was 1%, between Korean and European population, Korean and north eastern China.

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Table 3. Average distance among populations (%)

Gp1	0.6
Gp2	0.9
Gp3	0.1
Gp4	0.0
Gp5	n/c
Gp6	0.1
Gp7	0.1
Gp8	n/c

Table 4. Average distance between populations (%)

	Gp8	Gp3	Gp1	Gp2	Gp7	Gp6	Gp5	Gp4
Gp8								
Gp3	0.5							
Gp1	0.3	0.8						
Gp2	0.5	1	0.8					
Gp7	0.1	0.6	0.4	0.5				
Gp6	0.0	0.5	0.4	0.5	0.1			
Gp5	0.5	0.1	0.8	1	0.6	0.5		
Gp4	0.0	0.5	0.3	0.5	0.1	0.0	0.5	

Figure 2. A maximum likelihood tree with 53 complete sequences of mitochondrial 12S rRNA gene based on the Hasegawa-Kishino-Yano model [4]. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 54 nucleotide sequences. The tree was constructed with 1000 bootstrapped replications. *Camelus bactrianus* (AP003423) was used as out-group. Evolutionary analysis was conducted in MEGA 6 [12].

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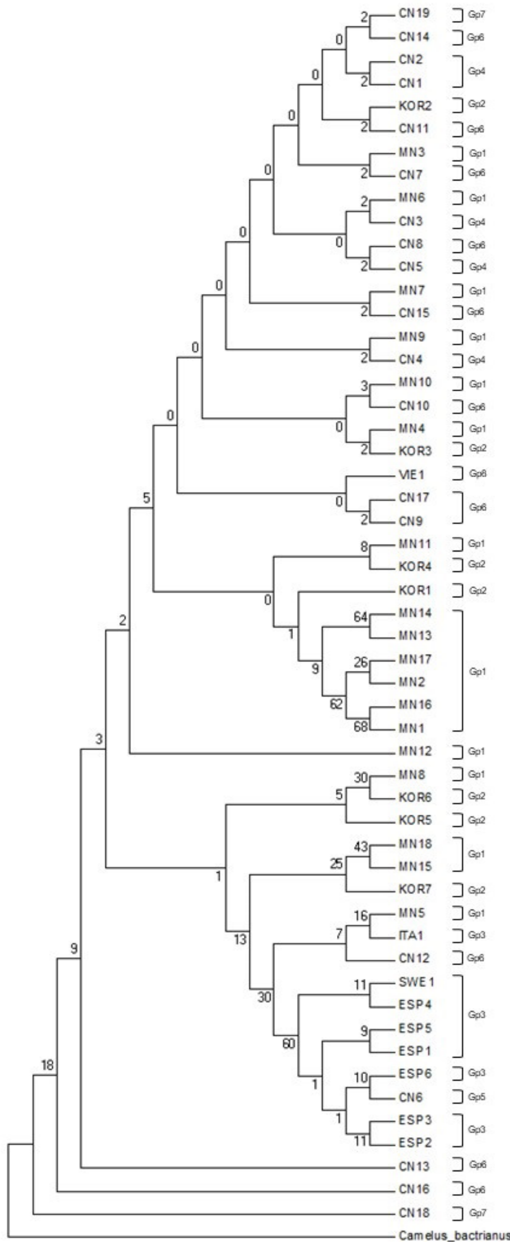

Figure 2. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model [4]. The bootstrap consensus tree inferred from 1000 replicates [2] is taken to represent the evolutionary history of the taxa analyzed [2]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 54 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 761 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [12].

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A maximum likelihood tree with 53 complete sequences of mitochondrial 12S rRNA gene of *Sus scrofa* from 4 major populations (Mongolia, China, Korea, Europe) comprising eight geographical locations (Gp1 to 8) are shown in Figure 2. Mongolian sequences from the same and adjacent locations were clustered together, such as MN13 and MN14, both from Uvs aimag, MN2 and MN17 Zavkhan aimag, MN1 and MN16 Khuvsgul aimag. European sequences (SWE1, ESP4, ESP5 and ESP1) were grouped, two sequences (CN1 and CN2) from south eastern China and two sequences (CN9 and CN17) from south western China were also grouped.

DISCUSSION

Wild boar populations are abundant and categorized as least concern in the world, and in Mongolia no data on population of wild boars are available at present. Based on both morphological and geographical differences, two sub-species, *Sus scrofa raddianus* and *Sus scrofa nigripes*, are distributed in Mongolia [7]. However, it is known that threats, particularly exploitation, hybridization and environmental degradation are having huge impact on population size of wild boars in Mongolia. Regionally, *Sus scrofa nigripes* may be re-categorized as threatened and *Sus scrofa raddianus* as near-threatened. Results of phylogenetic study of Mongolian wild boars will have impact on their future protection, rehabilitation and rational use.

In this study, 18 complete sequences of Mongolian wild boar mitochondrial 12S rRNA gene from 10 locations were compared to 35 complete sequences of mitochondrial 12S rRNA gene of wild boars available at NCBI GenBank. Average distance among populations (Table 3) in Asia ranged from 0% to 0.9% and in Europe the distance was 0.1%. Mongolian wild boar population distance was 0.6%, confirming sample collection was from all across Mongolia. Average distance between populations (Table 4) ranged from 0% to 1%. Average distance between Mongolian and other populations ranged from 0.3% to 0.8%, suggesting Mongolian population was located in-between populations.

A maximum likelihood tree with the 53 complete sequences of mitochondrial 12S rRNA gene of *Sus scrofa* was constructed with 1000 bootstrapped replication based on Hasegawa-Kishino-Yano (HKY) model. As expected, sequences from same locations were grouped in the constructed tree, suggesting individuals from same location were identical in their sequences. Mongolian sequences from Uvs (MN13 and MN14), Zavkhan (MN2 and MN17) and Khuvsgul (MN1 and MN16) aimags were grouped. Moreover populations from Europe, south western China and south eastern China were grouped. Additionally, adjacent populations from south eastern (Gp4) and south western (Gp6) China, south western (Gp6) and southern (Gp7) China branched close to each other. Adjacent populations from Khovd (MN18) and Uvs (MN15) aimag in Mongolia were close to each other. These results confirm the possibility of gene flow between populations.

Individual sequences of Mongolian wild boars were not grouped, thus we believe natural barriers, which limit species' distribution, played an important role in Mongolian wild boar populations. However, these sequences were close to each other. Sequences from adjacent aimags such as Khovd and Uvs were close to each other, but distant from other sequences from Mongolia. In the western part of Mongolia, especially Khovd and Uvs aimags, are inhabited by *Sus scrofa nigripes*, while *Sus scrofa raddianus* inhabits the northern and eastern part of Mongolia. The constructed phylogenetic tree confirms the morphological data of Mongolian wild boars.

Results of the study showed that Mongolian wild boar population was located in-between

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Asian and European populations. Sequences from Khovd and Uvs aimags were close to European populations, whereas Mongolian populations from other locations were close to Asian populations. Geographically, Khovd and Uvs aimags are in adjacent locations in the western part of Mongolia, close to the Russian border. Therefore, gene flow through Russia from European wild boar populations to Mongolian population and vice versa is possible. Gene flow between Asian populations and western Mongolian population was limited due to natural barriers such as the Gobi desert and the Altai mountain ranges. On the other hand, gene flow between other Mongolian populations is not restricted by natural barriers and the gene flow could be possibly through Russia and China.

CONCLUSIONS

In conclusion, the results of the study showed that geography plays a major part in *Sus scrofa* populations. Geographical location of Mongolia enabled gene flow between Asian and European *Sus scrofa* population. Using results of this study, it is possible to rehabilitate, regionally, threatened *Sus scrofa nigripes* and near-threatened *Sus scrofa raddianus* from adjacent populations.

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