PMAS
Proceedings of the Mongolian Academy of Sciences
Vol. 62 No 03 (243) 2022
DOI: https://doi.org/10.5564/pmas.v62i03.2472
ARTICLE

Phylogenetic analysis of mitochondrial D-loop sequence of Mongolian wild boars

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ARTICLE INFO: Received: 04 Aug, 2022; Accepted: 26 Dec, 2022

Abstract: Genomic DNA was extracted from tissue specimens of wild boars (*Sus scrofa*) in 10 different locations Mongolia. D-loop part of genome was amplified by PCR and was subjected to DNA sequencing. Determined sequences from 18 specimens were registered with the GenBank and accession numbers were obtained. A total of 54 complete mitochondrial D-loop sequences of wild boars available with NCBI GenBank were taken as a reference for comparison with that of Mongolian wild boars. Sequence alignment, detection of parsimonious informative sites, model selection, calculation of nucleotide distances, and Maximum Likelihood (ML) phylogenetic tree construction with 1000 bootstrapped replications were conducted using MEGA X software. Maximum Likelihood trees were constructed by the Hasegawa-Kishino-Yano (HKY) model. The results of the study showed that geographic location played an important role in sequence divergence between wild boars from various locations. Most of them were grouped together according to their respective geographic locations, except for several individuals. It is highly likely that the Mongolian subpopulation of wild boars, such as *S. scrofa raddianus* and *S. scrofa nigripes*, have had the same ancestor. In order to fully evaluate the distribution, ecology, and biology of Mongolian wild boars, it is essential to compare supplemental gene sequences that can reveal phylogenetic differences from the populations in the neighboring areas, such as Russia, northeast China, and Kazakhstan. The results of this study will be useful and informative for the protecting and conserving of wild boars in Mongolia.

Keywords: *Sus scrofa; D-loop; phylogenetic analysis; mitochondrial DNA;*

INTRODUCTION

The wild boar Sus scrofa occurs in a wide area of Asia, Europe and North Africa. Human activity played a major role in the expansion of its habitat. Four subspecies groupings of wild boar are distinguished according to both geographic and morphological criteria [3]

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DOI: https://doi.org/10.5564/pmas.v62i03.2472

Two subspecies inhabit Mongolia today: the *Sus scrofa nigripes* in the forested regions of western Mongolia, including the Great Lake Depression and the western Mongolian Altai Mountain Ranges; while the *Sus scrofa raddianus* occurs in eastern and northern regions of Mongolia, including in the Khangai, Khuvsgul and Khentii mountain ranges, Ikh Khyangan Mountain ranges and the Mongol Daguur Steppe [7].

The wild boar populations are one of the widest-ranging species and are listed as "Least Concern" globally by the IUCN, however, in Mongolia today data on the population of wild boars are not available.

Threats such as exploitation, hybridization, and environmental degradation are having a huge impact on the size of the population of Mongolian wild boars [8]. Regionally, *S. s nigripes* can be categorized as threatened and S. s raddianus as near threatened by the IUCN. Since wild boars are susceptible to a variety of highly contagious diseases, which are the main causes resulting in the shrinking of their population [9].

In this study, the nucleotide sequences of mtDNA control regions of Mongolian wild boars, collected from several locations, were identified for investigating the relationship between these two subspecies and other wild boar populations in the world.

MATERIALS AND METHODS

The tissue samples from 18 Mongolian wild boar (*Sus scrofa nigripes* and *Sus scrofa raddianus*) individuals were collected from ten different locations (Fig. 1) around the country. Survey samples of biomaterial were collected by making requests from economic entities and citizens who possess relevant hunting permits. The obtained nucleotide sequences were compared with 54 complete sequences of mitochondrial D-loop of *Sus scrofa* (Table 1) from two different locations in Europe, four different locations in Asia, and one location in Africa, including the outgroup sequence available at NCBI GenBank.

The collected tissue samples were either kept under cold conditions or in ethanol until delivery to the laboratory. Samples were stored at a temperature of -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 were subject to vigorous shaking at 55°C for 2-3 hours. DNA was extracted with an equal volume of phenol: chloroform: isoamyl alcohol and precipitated with 2.5 volumes of 96% cold ethanol and 0.1 volume of 3 M acetate Na. The integrity of isolated DNA was run on 1.2% agarose gel electrophoresis and the purity was determined basing on the optical density ratio at 260:280 nm.

D-loop was PCR-amplified using the following primer pairs: forward primer 5'–CGCCATCAGCACCCAAAGCT–3' and reverse primer 5'–GGAGCTGTGAGGCTCATCTAG–3'. The reaction was set up in 0.2 ml PCR tubes containing 10X buffer 2 µl, 200 mM dNTPs, 1 µM 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. The final extension was carried out at 72°C for 10 min. The PCR products, run on 1.2% agarose electrophoresis, were subject to DNA sequencing. The result of DNA sequences was registered to GenBank and was granted accession numbers.

The mitochondrial D-loop sequences and accession numbers from Mongolian S. scrofa are depicted in Table 1. The complete mitochondrial D-loop sequences were obtained from 18 specimens at 10 locations in Mongolia (Fig. 1) and analyzed together with complete sequences of mitochondrial D-loop of S. scrofa (Table 2) that are available at NCBI GenBank.

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Table 1. The location and coordinates for sampling, sequence name and accession numbers for the D-loop sequence from Mongolian S. scrofa

Sequence name	Location	Coordinates	GenBank accession number
MN1	Khuvsgul aimag	N51°25’32.15” E99°18’52.84”	KP027448
MN2	Zavkhan aimag	N48°28’20.86” E98°05’57.36”	KP027449
MN3	Arkhangai aimag	N48°35’13.33” E101°02’35.53”	KP027450
MN4	Tuv aimag	N48°06’05.09” E107°53’02.41”	KP027451
MN5	Khuvsgul aimag	N50°26’53.51” E101°40’33.27”	KP027452
MN6	Selenge aimag	N50°04’54.88” E106°11’44.43”	KP027453
MN7	Orkhon aimag	N49°05’02.66” E103°58’02.27”	KP027454
MN8	Uvs aimag	N49°05’04.50” E108°45’16.16”	KP027455
MN9	Uvurkhangai aimag	N46°35’57.48” E102°21’30.17”	KP027456
MN10	Dornod aimag	N49°03’41.51” E112°07’17.92”	KP027457
MN11	Arkhangai aimag	N46°03’53.06” E100°49’01.43”	KP027458
MN12	Khovd aimag	N47°54’17.36” E92°06’59.67”	KP027459
MN13	Uvs aimag	N50°33’53.22” E93°38’11.20”	KP027460
MN14	Uvs aimag	N50°20’12.16” E91°35’49.38”	KP027461
MN15	Uvs aimag	N49°28’27.01” E94°22’41.74”	KP027462
MN16	Khuvsgul aimag	N49°37’48.68” E102°00’34.85”	KP027463
MN17	Zavkhan aimag	N48°17’21.56” E98°15’16.15”	KP027464
MN18	Khovd aimag	N47°54’17.36” E92°06’59.67”	KP027465

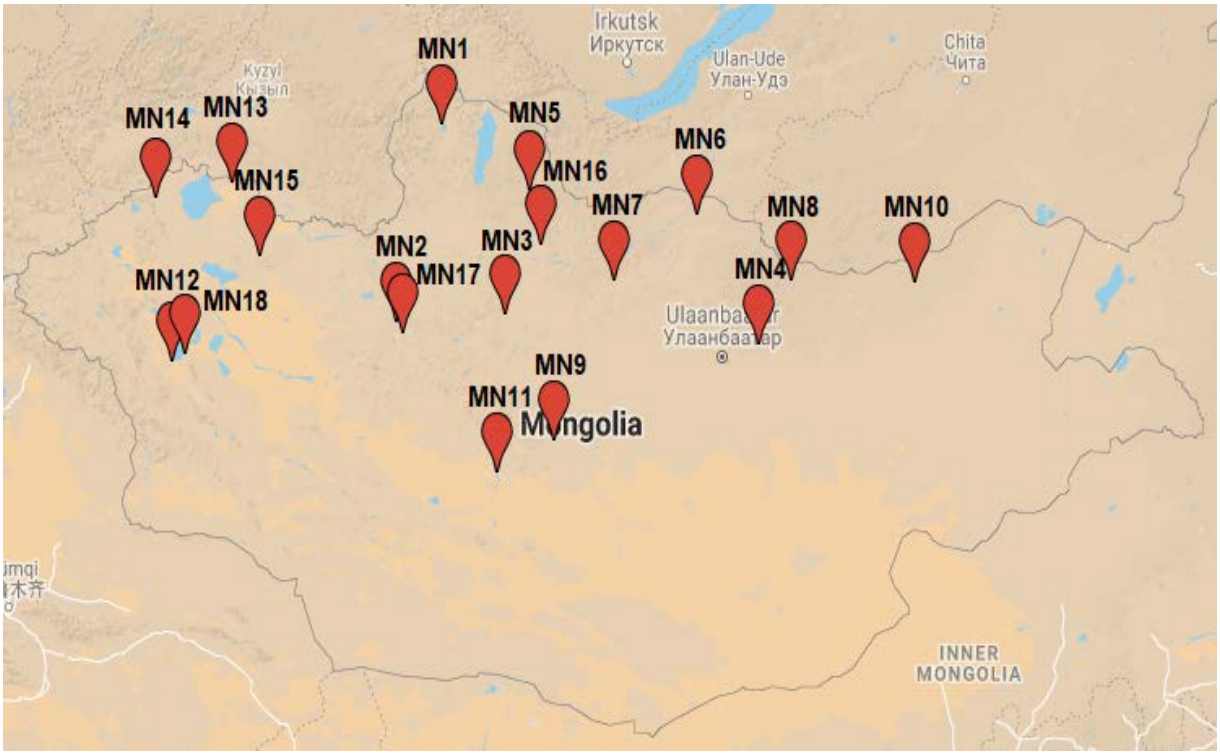

Figure 1. Sampling locations of 18 Sus scrofa specimens. Three specimens (MN1, MN5, MN16) from Khuvsgul aimag, two (MN2, MN17) from Zavkhan, two (MN3, MN11) from Arkhangai, one each (MN4 and MN6) from Tuv and Selenge aimags, four (MN8, MN13, MN14, MN15) from Uvs, one (MN7) from Orkhon, one each (MN9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

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Table 2. D-loop sequence data of wild boars obtained from NCBI GenBank. Three complete D-loop sequences from Europe (one from an unknown location in Europe and two from Italy), 45 complete sequences from Asia (20 from China, 11 from Japan, 14 from Korea), and five complete sequences from Africa (Morocco)

Region	Location	GenBank accession number	Sequence name
Europe	Italy	AB015094 AB015095 AB059651	EU-01 EU-02 EU-03
Asia	China	EF545586, EF545585, EF545568, EF545580, KC505411, EF545579, EF545572, EF545571, EF545570, EF545569	CN-01, CN-02, CN-03, CN-04, CN-05, CN-06, CN-07, CN-08, CN-09, CN-10
		AY486116, AY463062, MG837549, KM073256, KC493612, KC493611, KC493610, KC493609, KC493608, KC493607	TIB-01, TIB-02, TIB-03, TIB-04, TIB-05, TIB-06, TIB-07, TIB-08, TIB-09, TIB-10
	Korea	AY534288, AY534287, AY534286, AY534284, AY534282, AY534285, AY534283, EU090703, EU090702, AY574047, DQ268530, DQ207753, DQ207755, DQ207754	KOR-01, KOR-02, KOR-03, KOR-04, KOR-05, KOR-06, KOR-07, KOR-08, KOR-09, KOR-10, KOR-11, KOR-12, KOR-13, KOR-14
	Japan	AB015084, AB015085, AB015086, AB015087, AB015088, AB015089, AB015090, AB041473, AB041472, AB041471, D42184	JAP-01, JAP-02, JAP-03, JAP-04, JAP-05, JAP-06, JAP-07, JAP-08, JAP-09, JAP-10, JAP-11
Africa	Morocco	KU664558, KU664557, KU664556, KU664555, KU664554	MOR-01, MOR-02, MOR-03, MOR-04, MOR-05

Complete D-loop sequences of Mongolian wild boars were analyzed together with a sequence of the same region from 54 individuals, including an out-group from two locations in Europe, four locations in Asia and one location in Africa. The obtained nucleotide sequences were edited and aligned with ClustalW, MEGA X [6]. The parsimonious informative sites detection, model selection, nucleotide distance calculation, and phylogenetic tree was conducted using MEGA X with 1000 bootstrap replicates [5]. The Hasegawa-Kishino-Yano (HKY) model [5], which showed the lowest Bayesian information criterion score was chosen as the best model for our data by the software. We decided to choose an out-group. The Camelus bactrianus (NCBI accession number AP003423) was taken as an out-group, since it was in the same order as *Sus scrofa* and it was not closely or distantly related to *Sus scrofa*.

RESULTS AND DISCUSSION

The phylogenetic analysis allowed to determine the relationship between wild boars from Mongolia and from different locations. In total, complete D-loop sequences from 71 individuals in six locations in Eurasia (Mongolia, China, Japan, Korea, and Europe) and one location in Africa (Morocco) were grouped together according to their geographical indication. The average evolutionary divergence of the D-loop sequence among groups showed that divergence between Eurasian groups was from

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0.33% to 0.62%. Divergence among individuals from Morocco was 1.49%, whereas the divergence from Mongolian individual was 2.2% (Table 3). The calculation results of D-loop sequence divergence showed that wild boars from different locations in Eurasia are mostly of the same species or subspecies. There is a possibility of *Sus scrofa algira* migration between populations in northern African countries [4]. Therefore, we assumed that migration caused relatively high divergence in D-loop sequences of Moroccan wild boar species. As for D-loop sequences, identified from two subspecies of Mongolian wild boars - *Sus scrofa nigripes* and *Sus scrofa raddianus*, their divergence was relatively high as compared to other groups.

Table 3. Estimates of average evolutionary divergence over D-loop sequence of wild boar pairs within groups

Region	Average divergence (%)
Mongolia	2.2
China	0.57
Japan	0.56
Korea	0.33
China Tibet	0.37
Europe	0.62
Morocco	1.49

Evolutionary divergence of the D-loop sequence amongst groups (Table 4) showed that divergence between Eurasian groups was from 0.55% to 1.48%. Divergence of the D-loop sequence of Japanese wild boar was relatively higher as compared to sequences of individuals from Eurasia. This can be related to the separation of Japanese islands from Eurasia in the Pleistocene epoch [10]. Geographical barriers played a major role in gene flow between wild boars from Eurasia and Japan. The divergence of Moroccan wild boars is highest compared to other groups. As depicted in the map (Figure 2) wild boars from northern Africa are separated from Eurasian wild boars by desert and sea. Among Eurasian wild boars, the divergence of Mongolian wild boars is the highest. The reason is the isolation of the wild boar population in Mongolia from the rest of other countries where wild boars are common.

Table 4. Estimates of evolutionary divergence over D-loop sequence of wild boar pairs between groups

Region	**Mongolia**	**China**	**Japan**	**Korea**	**Tibet**	**Europe**	**Morocco**
Mongolia							
China	3.92						
Japan	2.39	1.48					
Korea	2.19	0.55	1.23				
China Tibet	3.79	0.56	1.35	0.59			
Europe	2.29	0.73	0.89	0.79	0.72		
Morocco	6.63	4.00	5.48	4.13	4.13	4.47	

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Figure 2. Distribution map of wild boar [8]

The phylogenetic tree was constructed with nucleotide sequences of D-loop sequence from 71 individuals, including an out-group (Figure 3). Except for a few sequences, most D-loop sequences of wild boars were grouped according to their geographical location.

Mongolian wild boar D-loop sequences are located at the top of the phylogenetic tree. As expected, the Mongolian group is divided into two subgroups according to two subspecies of Mongolian wild boars, excluding sequence MN-05. The subgroup starting from sequence MN-17 to MN-02 comprised sequences obtained from the northern and eastern regions of Mongolia. The second subgroup with MN-16 to MN-12 includes sequences obtained from samples in the western part of Mongolia. The related taxa group, which is higher than 50%, indicates the same ancestor. As indicated in the phylogenetic tree the percentage between Mongolian wild boar subspecies was 76%. Based on these results, we concluded that the subspecies in Mongolia had a common ancestor.

Except for some sequences, D-loop sequences from Chinese provinces, including Tibet, are mixed. The percentage of grouping among these sequences is lower than 50%. The percentage of grouping among some D-loop sequences of Tibetan samples was higher than 50%. Except for some D-loop sequences from Chinese and European samples (EU-03), D-loop sequences of Tibetan samples were grouped together in the same clade.

D-loop sequences of Korean, European and Moroccan S. scrofa are grouped together according to their geographical location. The grouping percentage among the sequences from European samples was 97%, and that from Moroccan boars also showed a higher percentage, except MOR-03. Regarding D-loop sequences of Korean samples, the grouping percentage was mostly higher than 50%.

D-loop sequences from two subspecies (*Sus scrofa leucomystax* and *Sus scrofa riukiuanus*) of wild boars from Japan were used in this study. D-loop sequences from Japan are divided into two subgroups according to their subspecies. One subgroup, which is closer to Mongolian sequences, is comprised of D-loop sequences from *S. scrofa leucomystax*. The second subgroup, which consists of D-loop

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sequences from *S. scrofa riukiuanus*, is closer to the D-loop sequences of Europe, China, and Korea. Watanobe et al. (1999) hypothesized that Japanese wild boars originated from various geographic populations of continental wild boars. Similarities between Mongolian and Japanese wild boar *S. scrofa leucomystax* [11] can be seen in our phylogenetic tree.

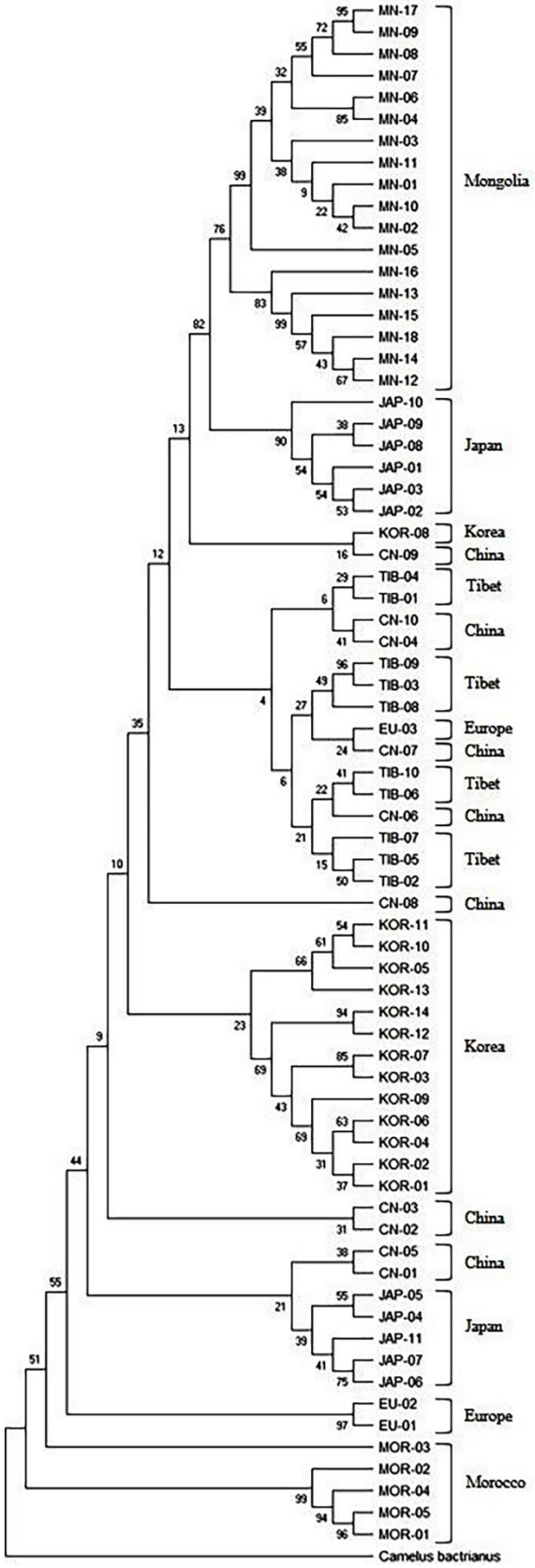

Figure 3. The evolutionary history was inferred by using the Maximum Likelihood method and the Hasegawa-Kishino-Yano model [5]. The bootstrap consensus tree inferred from 1000 replicates [2] is taken to represent the evolutionary history of the taxa analyzed [2]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. 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 automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with a superior log-likelihood value. This analysis involved 72 nucleotide sequences. There were 1464 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [6]

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Obtained results showed that Mongolian wild boar subspecies *S. scrofa nigripes* and *S. scrofa raddianus* most likely had a common ancestor. Geographical barriers, such as the Altai mountain range in the west and the Gobi desert in the south, isolated the Mongolian wild boars population from Eurasian wild boars. Thereby, D-loop sequences from Mongolian wild boars are distant from the sequences used in this study. The use of mitochondrial gene sequences from adjacent wild boar populations will be helpful in further determining the phylogenetic relationship and migration of wild boars.

In our joint research with the Korean group, the haplotypes we found supported the hypothesis of migration of wild boars from South-East Asia to South Asia, followed by migration to East and West Asia [1]. In that study, we included only 6 samples from Mongolia. To expand the obtained results and to verify the hypothesis, we analyzed haplotype distribution in 84 wild boar samples from Asian regions. Out of them, 18 samples were from Mongolia. The remaining were taken from data that were used in our previous study [1]. In total, 38 haplotypes of the mtDNA control region were identified in these samples (Table 5). The present results also confirm previous findings (Figure 4).

Table 5. Sample location and haplotype distribution of wild boar (S. scrofa) from 8 regions

Location	mtDNA control region		
	N	h	Haplotype
China	10	6	**Hap-33** (CYN-05), **Hap-34** (CYN-04), **Hap-35** (CYN-02, CYN-03), **Hap-36** (CYN-01), **Hap-37** (CXJ-02, CXJ-03, CXJ-04, CXJ-05), **Hap-38** (CXJ-01)
Estonia	6	2	**Hap-31** (EST-03, EST-04, EST-05, EST-06), **Hap-32** (EST-01, EST-02)
Indonesia	10	6	**Hap-25** (IND-07, IND-08, IND-09, IND-10), **Hap-26** (IND-06), **Hap-27** (IND-04, IND-05), **Hap-28** (IND-03), **Hap-29** (IND-02), **Hap-30** (IND-01)
Japan	10	3	**Hap-22** (JPN-08, JPN-10), **Hap-23** (JPN-05, JPN-06, JPN-07, JPN-09), **Hap-24** (JPN-01, JPN-02, JPN-03, JPN-04)
Korea	10	2	**Hap-20** (KJJ-01, KJJ-02, KJJ-03, KJJ-04, KJJ-5), **Hap-21** (KGG-01, KGG-02, KGG-03, KGG-04, KGG-05)
Mongolia	18	12	**Hap-8** (MN-15, MN-15), **Hap-9** (MN-17), **Hap-10** (MN-16), **Hap-11** (MN-14), **Hap-12** (MN-13), **Hap-13** (MN-12), **Hap-14** (MN-03, MN-04, MN-06, MN-11), **Hap-15** (MN-10), **Hap-16** (MN-09), **Hap-17** (MN-07, MN-08), **Hap-18** (MN-05), **Hap-19** (MN-01, MN-02)
Russia	10	3	**Hap-5** (RUP-10), **Hap-6** (RUP-06, RUP-07, RUP-08, RUP-09), **Hap-7** (RUP-01, RUP-02, RUP-03, RUP-04, RUP-05)
Vietnam	10	4	**Hap-1** (VIE-10, VIE-09), **Hap-2** (VIE-06, VIE-07, VIE-08), **Hap-3** (VIE-03, VIE-04, VIE-05), **Hap-4** (VIE-01, VIE-02)
Total	84	38	

The haplotypes were obtained from the mitochondrial DNA control region. N-sample size; h-number of haplotypes.

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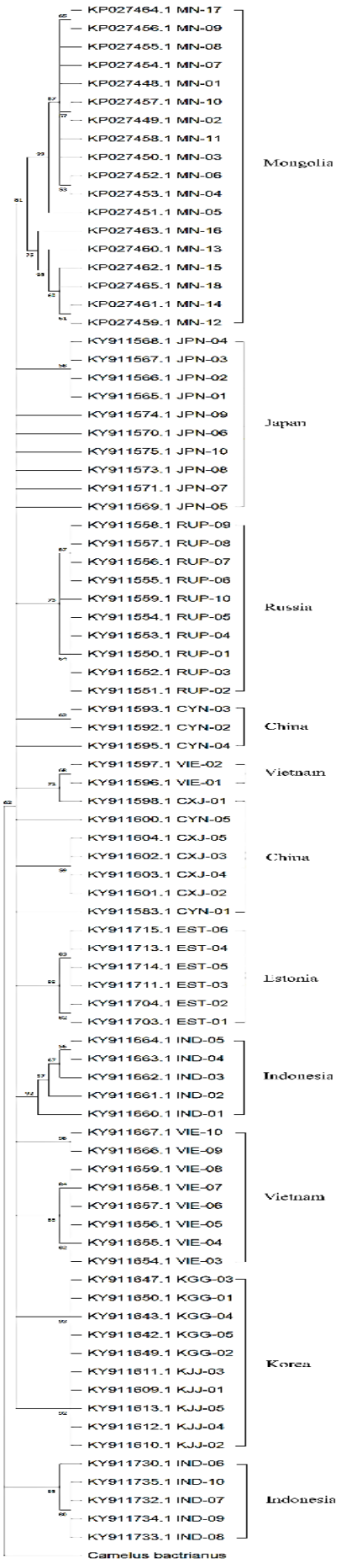

Figure 4. Haplotype tree constructed with the partial sequences of mtDNA control region of wild boar from Mongolia and from some other Asian regions through the Maximum Likelihood method and the Hasegawa-Kishino-Yano model with uniform rates

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CONCLUSIONS

Geographical isolation affected the divergence of wild boar populations in Mongolia. A comparative study of mitochondrial sequences from neighboring populations of wild boar in Russia, Kazakhstan and the northeastern part of China is needed for the investigation of phylogenetic relations and migration of wild boars.

Obtained results will be informative for the conservation of regionally threatened Sus scrofa nigripes and near-threatened Sus scrofa raddianus.

Acknowledgments: This research has been done within the framework of the project (P2021-4147) supported by the Mongolian Foundation for Science and Technology.

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