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ARTICLE

Genetic comparison of Altai and Gobi argali sheep (*Ovis ammon*) populations using mitochondrial and microsatellite markers: Implication on conservation

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Abstract: *Argali sheep is an ungulate, which inhabits the north, west, south and central regions of Mongolia. There are two major populations (Altai and Gobi) in Mongolia, but their taxonomic classification as subspecies is often disputed among researchers. Furthermore, there is no recent study about the population genetic structure of argali sheep in Mongolia. In the present study, we have investigated genetic diversity and difference between Altai and Gobi argali populations using mitochondrial control region hyper variable segment (HVS) sequence (598bp) and 3 microsatellite markers. Mitochondrial HVS haplotype analysis showed high haplotype diversity (0.982±0.012) and low nucleotide diversity (0.02589). In microsatellite analysis, total of 9 alleles were found across all loci while mean Ho were 0.59±0.13 for Altai and 0.53±0.1 for Gobi populations, indicating low allelic diversity with moderate heterozygosity. Neighbor-joining tree separated haplotypes into two clusters, Altai and Gobi population, implying distinct genetic difference between the two subspecies. Additionally, Pairwise F_{ST} and Kimura-2 parameter showed 0.127 and 0.0413±0.0068, respectively. These genetic distance analyses hinted genetic difference between Altai and Gobi populations are in subspecies level. In summary, mitochondrial HVS and microsatellite analysis demonstrated that Altai and Gobi populations had low genetic diversity but might be genetically distinct from each other in subspecies level, suggesting conservation should be separately managed.*

Keywords: *argali sheep; mitochondrial DNA; microsatellite; Ovis ammon; population genetic structure;*

INTRODUCTION

Ovis ammon, commonly known as argali sheep, is a species of wild sheep that mostly found on desert hills, plateaux and mountain slopes in Asia [1]. In Mongolia, two subspecies of argali sheep are commonly identified, *O. a. ammon* and *O. a. darwini*. These were first classified based on morphological features and later supported by genetic analysis studies [2], [3], [4]. *O. a. ammon* is distributed through Altai Mountains in Mongolia and neighboring regions of Russia, China and Kazakhstan [5]. *O. a. ammon*, known as Altai argali is the world's largest wild sheep. Rams are almost twice bigger than ewes and have huge curled

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horns, so it's often susceptible to trophy hunting [1]. Conversely, *O. a. darwini* (Gobi argali) is relatively smaller than *O. a. ammon* (Altai argali) in size and can be recognized by its tear shaped cross-section of horn base [2], [6]. Gobi argali inhabits mountains, rolling hills, rocky outcrop, valleys and steppes of Gobi Desert in southern regions of Mongolia [7]. Although these two subspecies may be morphologically and genetically distinctive, they are phylogenetically closer to each other than other subspecies of *O. ammon* [2]. Additionally, absence of major barriers between the regions enables free movement from Altai mountains to Gobi Desert via Trans-Altai Mountains [4]. Therefore, argali sheep is spreading more to the eastern side of the Mongolia and western populations are getting fragmented due to poaching and competition with livestock [8]. The present study has investigated *O. ammon* Altai and Gobi population structure by mitochondrial control region hyper variable segment (HVS) and microsatellite markers to investigate genetic diversity and difference between those argali sheep populations.

MATERIAL AND METHODS

Sample Collection and DNA isolation

We collected 50 argali sheep samples including blood, skin and pellets from Altai and Gobi regions. Gobi region includes Umnugobi and Dornogobi while Altai region includes Khovd, Uvs and Bayan-Ulgii. Total genomic DNA was extracted from blood and skin using QIAmp DNA kit (Qiagen) and from pellets using a DNA extraction method based on salting out method from Caprinae feacal samples [9].

Mitochondrial HVS PCR amplification and sequencing analysis

HVS was amplified using i-Taq DNA Polymerase (iNtRON Biotechnology) according to the manufacturer's instructions and sent to sequencing in Macrogen Inc. (South Korea). Primers for HVS of mtDNA were designed by comparing sequences of other subspecies belonging to *O. ammon* (Accession NOs: KX609626, AF242347, AY091492, JX101654.1) using software SnapGene and MEGA X [10], [11]. HVS was amplified and sequenced using following primers: Forward 5'-ACATACAACACGGACTTCCCAC-3' and Reverse 5'-GAGATGGCCCTGAAGAAA GAACC-3'.

Sequencing data were edited and aligned using software MEGA X and Bioedit [10], [12]. 598bp fragment of HVS was used for analysis. The parameters of the mitochondrial polymorphisms were computed using DnaSP version 5.10.01 [13], including segregation site (S), number of haplotypes, haplotype diversity (Hd), nucleotide diversity (π), average number of nucleotide differences (k). The neighbor-joining trees based on haplotypes of HSV were built using Tamura-Nei model by MEGA X [14]. Genetic distance based on HSV between Altai and Gobi population were calculated using Kimura-2 parameter (K2P) by the MEGA X software program [15].

Microsatellite genotyping and data analysis

We screened 7 pairs of primers from microsatellite markers developed for mountain ungulates in 5 individuals and three (B*M302, OarFCB20* and *INRA005*) of them were found as polymorphic [16]. These primers were used to amplify the polymorphic alleles using i-Taq DNA polymerase (iNtRON Biotechnology, South Korea) according to the manufacturer's instruction. Then, the PCR products were separated on 10% polyacrylamide gel electrophoresis and visualized by Silver Staining. In brief, the gel was treated with 1% HNO_3 for 10 min and then, stained with 0.1% $AgNO_3$ for 20 min. After rinsing with distilled water, the gel was incubated with 3% Na_2CO_3 and 37% formaldehyde until the band was visible. The gel was analyzed by Gel Doc EZ Imager (Bio-Rad Laboratories, USA) and its' software. Sizes of the bands were estimated

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using Sizer™-50 plus DNA Marker Solution (iNtRON Biotechnology, South Korea) as reference and genotyped according to the fragment size.

Allele frequencies, observed (Na) and effective number of allele (Ne), expected (He) and observed heterozygosity (Ho) for each locus were calculated using GenAIEx 6.503 software [17]. Tests for Hardi-Weinberg Equilibrium (HWE) by population-locus and Pairwise FST between the populations were also estimated by GenAIEx 6.503 software.

RESULTS AND DISCUSSION

Mitochondrial HVS diversity

Mitochondrial 598bp fragment of HVS was successfully amplified and sequenced in total of 30 individuals (20 from Altai and 10 from Gobi populations). The haplotype analysis detected 38 variable positions defining 22 different haplotypes (H1-H22), of which 15 appeared from Altai and 7 appeared from the Gobi. 14 haplotypes (H2-H3, H5-H6, H12, H14-H22) were in one individual, while other 8 haplotypes (H1, H4, H7-H8, H9-H11, H13) were found in 2 individuals (Supplementary, Table 1). All haplotypes dispersed across the populations with average frequency 3.33%-6.66%, which are quite low. Low haplotype frequency indicates mutation have been recently created for evolutionary purposes. On the other hand, it is perhaps the numbers of the populations studied were low and their distribution has been fragmented [18], [19].

In Altai population, π and k were lower than Gobi population, but Hd was higher than Gobi population (Table 1). Overall Hd and π were 0.982±0.012 and 0.02589, respectively. These results demonstrated Altai and Gobi populations have low genetic diversity with high genetic differentiation, hinting the subpopulations within these two populations have been isolated from each other and may lead to reduction in population size [20], [21].

Table 1. The segregation site (S), number of haplotypes, haplotype diversity (Hd), nucleotide diversity (π), average number of nucleotide differences (k) for each population based on mitochondrial HVS sequences

Populations	S	Number of haplotypes	Hd	π	k
Altai (n=20)	31	15	0.974±0.022	0.01347	8.05789
Gobi (n=10)	38	7	0.933±0.062	0.01847	11.04444

Microsatellite diversity

Microsatellite genotype data at 3 loci were obtained from a total of 37 individuals (18 from Altai and 19 from Gobi). In total, 9 alleles were detected; 3 alleles from each locus were observed in both populations except 2 alleles were spotted in Gobi population on OarFCB20 locus. The frequencies of the alleles in each locus are shown in Table 2.

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Table 2. Observed alleles and their frequencies of BM302, OarFCB20 and INRA005 loci in Altai and Gobi populations

Population	Loci					
	BM302		OarFCB20		INRA005	
	Allele (n=3)	Allele Frequencies	Allele (n=3)	Allele Frequencies	Allele (n=3)	Allele Frequencies
Altai (n=18)	A	0.139	A	0.500	A	0.083
	B	0.639	B	0.361	B	0.778
	C	0.222	C	0.139	C	0.139
Gobi (n=19)	A	0.605	A	0.368	A	0.579
	B	0.342	B	0.632	B	0.342
	C	0.053	C	None	C	0.079

The Na, Ne, Ho and He of each marker in both populations are shown in Table 3. The Ne was ranged from 1.58 (INRA005 locus) to 2.5 (OarFCB20 locus), but the mean Ne of the populations were similar, 2.06±0.26 (Altai) and 2.03±0.09 (Gobi) respectively.

Na in each locus was quite low compared to the previous study [3], suggesting decrease in the allelic diversity. Allelic diversity is an indicator of population's potency for adaptation to future environmental changes [22]. Consequently, the reduction in allelic diversity may have negative impact on its survivability.

Table 3. Observed (Na) and effective number of allele (Ne), expected (He) and observed heterozygosity (Ho) of BM302, OarFCB20 and INRA005 loci in Altai and Gobi populations

Locus	BM302				OarFCB20				INRA005			
	Na	Ne	Ho	He	Na	Ne	Ho	He	Na	Ne	Ho	He
Altai (n=18)	3	2.09	0.72	0.52	3	2.5	0.72	0.6	3	1.58	0.33	0.37
Gobi (n=19)	3	2.06	0.37	0.52	2	1.87	0.53	0.47	3	2.18	0.68	0.54

However, heterozygosity analysis suggested otherwise. Based on Table 3, the mean Ho of the population were 0.59±0.13 (Altai) and 0.53±0.1 (Gobi) while the mean He were 0.49±0.06 (Altai) and 0.51±0.02 (Gobi). The Ho were similar to previous study [3], and mean heterozygosity was higher than 0.5 value, suggesting average heterozygosity and safe from inbreeding depression [23], [24].

Genetic difference between Altai and Gobi populations

The genetic difference between the two populations has been calculated based on HVS sequence and microsatellite alleles. By using neighbor-joining, all HVS haplotypes were divided in two clusters. Haplotype H2 and H22, belonging to Altai population, was clustered together with haplotypes from Gobi population while H19 from the Gobi population, was grouped with haplotypes from Altai population (Fig.1). The mis-grouping could have happened due to migration between the populations, as there are no substantial obstacles between them [4].

Additionally, HWE was tested in each locus on both populations and significant deviations ($p<0.05$) were found in INRA005 and OarFCB20 loci in Altai population while only INRA005 locus in Gobi population. The deviations from HWE can be due to the high number of heterozygote individuals compared to the homozygous one's migration and mutation rate of the loci [25], [26]. HWE exhibits population's ability to preserve

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it's allelic frequencies but crossbreeding between the populations can change the allelic frequencies and deviate from HWE. Yet, Pairwise F_{ST} across the all loci was 0.127, indicating moderate genetic difference between the population. Furthermore, K2P genetic distance based on HVS sequences was 0.0413±0.0068 between Altai and Gobi populations. Total number of mutations is 38, of which 36 (94.7%) were transitions, and 2 of them were transversion.

The K2P results were consistent with other studies done between sheep subspecies, suggesting differentiation in subspecies level [27], [28]. So, haplotype grouping misplacement and the deviation from HWE might be related to the movement between the populations [4], [29]. These results indicate genetic difference between the two subspecies, *O. a. ammon* from Altai and *O. a. darwini* from Gobi [2], [4].

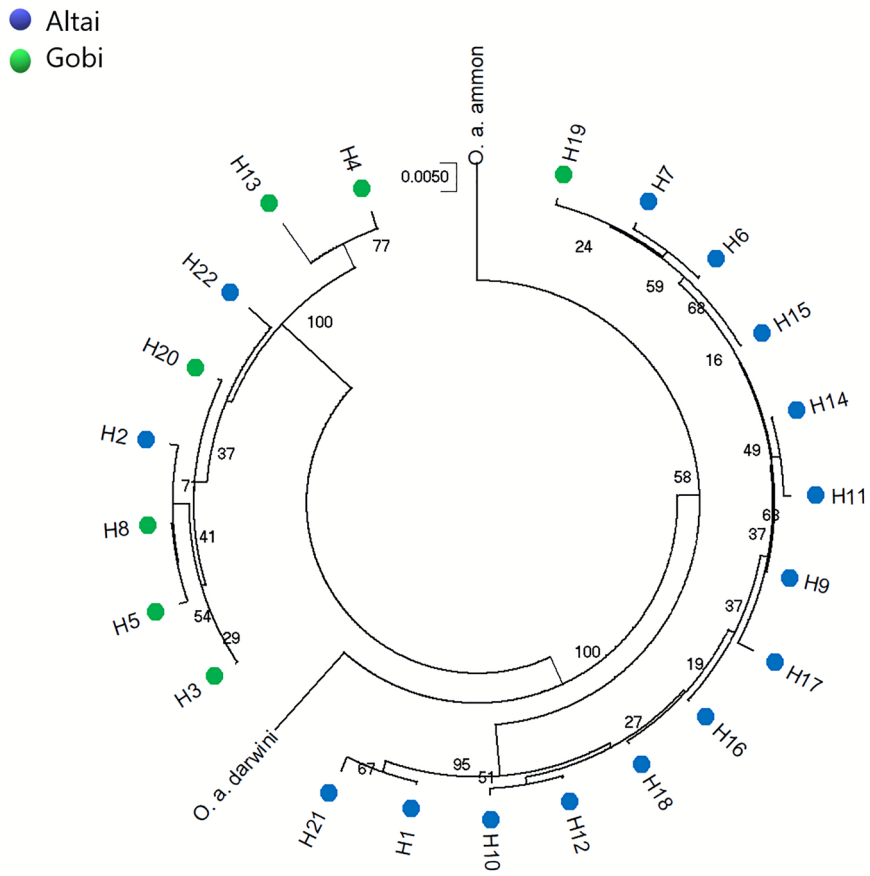

Figure 1. Phylogenetic tree based on haplotypes (598-bp) of mtDNA using neighbor-joining method and Tamura-Nei model. The Haplotype from Altai was marked with Blue color, while haplotype from the Gobi was marked in Green. Numbers in nodes denote bootstrap value with 1000 replications. O. a. ammon and O. a. darwini were used for reference sequences (Accession no: AF242347, KX609626)

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Implication in Conservation

Argali sheep is classified as Endangered in Mongolian Red List of Mammals and hunting has been prohibited except for the annual quota of trophy hunting [8], [30]. Our results divided HVS haplotypes into two groups, Altai argali and Gobi argali. There were some haplotypes that got mis-grouped, suggesting free movement between the populations. The genetic differentiation analysis, K2P of mitochondrial HVS and Pairwise FST analysis using microsatellite markers indicated distinct differentiation of Altai and Gobi populations, suggesting argali sheep from these two populations to be separated as two evolutionary significant units (ESU) in conservation management [3], [4].

Our mitochondrial HVS results showed Altai and Gobi populations have low genetic diversity and high genetic differentiation. Microsatellite analysis also supported those results, showing Altai and Gobi populations have low genetic diversity with moderate heterozygosity. These results indicate Altai and Gobi populations might be becoming a population containing isolated small subpopulations due to habitat fragmentation. Habitat fragmentation causes increased genetic drift and decreased gene flow, which leads to loss of genetic diversity within the fragmented subpopulation and the increase of genetic differentiation between those subpopulations [31].

The fragmentation of Altai and Gobi populations might be mainly due to competition with livestock, poaching, and human disturbance [5]. One possible solution is to connect the fragmented subpopulations by preserving or building corridors, such as bridge or underpass. Another way is to encourage cooperation between rangers of Protected Areas (PAs) and local herders to improve their awareness about the negative impact of livestock overpopulation on argali sheep reservation. Moreover, Altai argali is vulnerable to poaching due to ram's huge size [8].

Except the annual quota of trophy hunting, argali sheep from Altai and Gobi populations are often illegally hunted, which may decrease the number of individuals in the populations, and consequently cause inbreeding [8]. Moreover, hunting regions adjacent to the PAs, affect negatively on protection of argali sheep. Our results showed low genetic diversity of argali sheep, suggesting until stabilization of the population diversity, hunting should be limited as much as possible to minimum. In addition, to recover distribution of Altai and Gobi populations, there might be the need of establishing new PAs in other regions, where majority of the population inhabits. At least, 20 percent of the distribution area of argali sheep in Mongolia is suggested to be taken as PAs.

CONCLUSIONS

In conclusion, Altai and Gobi population of argali sheep had low genetic diversity and although there may have been some mixture of individuals between the populations, the distinctiveness between the populations still exist. The current study could show good conservation clue, though it is suggested to increase the sample size for further precise conclusions.

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