Original Article

Genetic Structure of Mongolian Gazelle (*Procapra gutturosa*): The Effect of Railroad and Demographic Change

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Abstract

The Mongolian gazelle (*Procapra gutturosa*) is a representative ungulate species of Mongolia that inhabits steppes. Their number and range decreased during the last century, and the population has been suffered from occasional demographic changes caused by human and environmental factors. During the summer of 2005, we obtained genetic samples from gazelle carcasses encountered along the international railroad between Russia and China, to examine genetic diversity and its changes in relation to historical demographic shifts. Gazelle genetic structure and diversity were investigated using mitochondrial control region sequence. In the phylogenetic analysis, we confirmed that there are two genetic groups unrelated to geographical location. We also showed the genetic structure of gazelles was unrelated to existence of the railroad. Based on the genetic diversity indices and demographic parameters, the population was suggested to have experienced demographic expansion historically, and effect of known demographic decline was not detected.

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Introduction

Mongolian gazelles (*Procapra gutturosa*) a migratory antelope species; they are recognized as one of the largest remaining wildlife populations in Asia. During 1950s, Mongolian gazelles widely distributed in steppe and semi-desert ecosystems of 780,000 km² range throughout Mongolia, parts of Kazakhstan, the Russian Federation, and in China (Bannikov *et al.,* 1961; Lhagvasuren & Milner-Gulland, 1997). In the past 50 years, however, their entire range

reduced to 190,000 km² (Lhagvasuren & Milner-Gulland, 1997; Milner-Gulland & Lhagvasuren, 1998), and the present distribution is restricted to the eastern part of their original range, mainly in the Dornod, Khentii, Sukhbaatar, and Dornogobi provinces, with several small isolated herds in western Mongolia (Lhagvasuren & Milner-Gulland, 1997).

Mongolian gazelles experience periodic catastrophic declines that can potentially affect on genetic diversity of the species. Milner-Gulland & Lhagvasuren (1998) estimated more than 4.5 millions gazelles existed in the beginning of 20th century. However, the population declined rapidly from 1979 to 1986, with slight increase afterwards (Lhagvasuren & Milner-Gulland, 1997). The recent population estimates of gazelles suggest 2.7 million in 1994 (unpublished air survey; Milner-Gulland & Lhagvasuren, 1998), and approximately 1.1 million individuals within a 275,000 km² portion of the Eastern steppe of Mongolia (Olson, 2010; Olson *et al.*, 2011). Although the global status of the Mongolian gazelles is the Least Concerned in the International Union for Conservation of Nature Red List (IUCN 2009), they are categorized as endangered regionally (Clark *et al.*, 2006), mainly due to illegal hunting, as well as other factors including harsh climate, disease, and competition with livestock for resources, and habitat fragmentation (Lhagvasuren & Milner-Gulland 1997; Milner-Gulland & Lhagvasuren 1998; Jiang *et al.*, 1998; Reading *et al.*, 1998; Leimgruber *et al.*, 2001; Campos-Arceiz *et al.*, 2004). The international railroad between Russia to China established during 1965s bisects the gazelle habitat, and it appears to influence on gazelle movement and survival (Ito *et al.*, 2005, 2006, 2008).

Species history related to changes in demography or spatial distribution affects the frequency and the spatial pattern of alleles. When the population lacks of the genetic diversity, it loses the potential for adaptation in the natural selection (Mills, 2007). The gazelles, being suffered from population decline or the habitat fragmentation through their history, would have lost genetic diversity through drift. In this study we obtained tissue samples of more than a hundred Mongolian gazelle carcasses along the international railroad in 2005 (*see* Ito *et al.*, 2008), and investigated their genetic structure.

A previous genetic study of Mongolian gazelles using samples from wide range, i.e., Mongolia, Russia, and China revealed there are two clear genetic lineages, and the structure was unrelated to geographical location (Sorokin *et al.*, 2005; Sorokin & Kholodova, 2006). We carried out similar phylogenetic analysis to confirm if the genetic structure in previous study was also seen in our samples, and also examined the influence of the railroad. Additionally, we calculated genetic diversity indices and demographic parameters, and examined the relationships of these values with population history of gazelles.

Material and Methods

Samples. We conducted a carcass survey of Mongolian gazelles from June to July 2005 along the international railroad from Ulaanbaatar to the Chinese border throughout the gazelle habitat as described (Ito *et al.,* 2008; Fig. 1). In the year there were many dead gazelles along the railroad, and we drove there and collected carcasses with dried skin. The railroad is fenced on both sides to prevent livestock accidents. Many of the gazelle carcasses were found outside of the fence, although some were inside the fenced area. Sex and approximate ages of carcasses were determined and the locations of each carcass were recorded using Global Positioning System (GPS). In addition, the side of the railroad on which the carcass was found was recorded, as well as whether it was inside or outside the fenced area.

The distribution of the samples was relatively continuous and covered a large area (> 500 km) from northwest to southeast. Therefore, for the analyses we divided the carcasses by whether they were north or south of the middle latitude of the sampling region (45˚75'N, 108˚40'E). Then, the samples were divided into groups according to whether they were in east or west side of the railroad. Therefore, the samples were categorized into four area groups: north of the middle latitude and from the eastern side of the railroad (NE), south of the middle latitude and from the eastern side (SE), north-western (NW), and south-western (SW). The samples collected from inside the fenced area were categorized into a group (IN).

Skin tissues, usually from ear, were collected for molecular analysis and preserved in 99.5 %
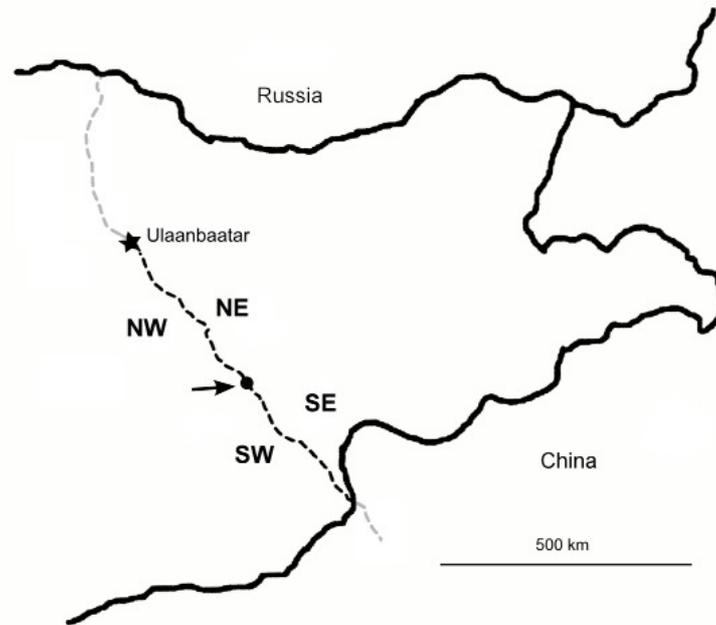
Figure 1. Map showing the central to eastern part of Mongolia. The samples were obtained along the railroad, ranging from Ulaanbaatar to Chinese border (dark dashed line), and the sampling area was divided into four at the middle-latitude sites (circle indicated by arrow) of the northern-most and southern-most sampling sites.

ethanol. DNA was extracted using the phenol-chloroform method according to Sambrook *et al.,* (1989).

Analysis of the control region. A hypervariable fragment of the mitochondrial DNA control region was used as a molecular marker. The following primer sets were designed on the basis of the reported control region of the gazelles and related species: forward 5'-CTTCAAGGAAGAAGCTATGGCT -3' and reverse 5'- GGTGATGCTCAAGATGCAGT -3'. For the nested PCR, the forward primer 5'- CACTATCAACACCCAAAGCTGAAG -3' designed at the San Diego Zoo Center for Reproduction of Endangered Wildlife (United States) (Sorokin *et al.,* 2005) and reverse primer 5'- GCCCTGAAGAAAGAACCAGATG -3' were used.

The initial PCR was carried out with 30 ng of template DNA in a 20 μl volume including 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP with 0.4 μM of each outer primer and 0.5 U Taq DNA polymerase (TaKaRa EX-Taq, TaKaRa Bio Inc., Japan), using a DNA thermal cycler (Thermal Cycler Dice Model 1600, TaKaRa Bio Inc., Japan). The following protocol was used: 3-min incubation at 94°C, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. When the product was observed in the gel

electrophoresis, 0.5 μl of the first PCR product was amplified for an additional 30 cycles using the inner pair of primers for the nested PCR and the same PCR profile. The nested PCR product was purified using Microcon™-100 column (Millipore, Bedford, MA, USA) according to manufacture's instructions. Each of the nested PCR products was sequenced using the primers for the nested PCR described above using an ABI 310 automated DNA sequencer and a BigDye v1.1 Cycle Sequencing Kit (Applied Biosystems).

The sequences were aligned using MUSCLE (Edgar, 2004) and MEGA4 software (Tamura *et al.,* 2007) using the default parameters. Neighbor-joining (NJ) trees were constructed using the default setting; except we used the substitutional model as the Kimura-2 parameter (Kimura, 1980; Saitou & Nei, 1987) as previous study (Sorokin *et al.,* 2005) had carried out. Bootstrap runs consisted of 1,000 replicates for each tree (Felsenstein, 1985). For each method, several sequences of the samples from Russia and China (Sorokin *et al.,* 2005), obtained from the GenBank/EMBL/DDBJ DNA database were included for comparison.

Using Arlequin 3.5.1.2 (Excoffier & Lischer, 2010), we calculated various molecular indices. As the genetic diversity indices, we used haplotype diversity (Nei, 1987) and nucleotide

diversity (Tajima, 1983). To estimate past demographic status we calculated Tajima's *D* (Tajima, 1989) and Fu's *FS* (Fu, 1997), which test the deviation from the neutral mutation hypothesis. Mismatch distribution analyses from the observed and expected pair-wise sequence differences based on the two models (demographic expansion model and spatial expansion model) were carried out and sum of squared deviations (SSD) (Schneider & Excoffier, 1999; Excoffier, 2004) was calculated for each analysis. Each value was tested by bootstrapping method (n = 1,000).

Results

Phylogeny of the sampled Mongolian gazelles. We collected 241 gazelle carcasses along the railroad. We successfully amplified part of the mitochondrial DNA (mtDNA) control region from 103 samples. The location of these carcasses were: 8 from the NE, 20 from the SE, 27 from the NW, and 23 from the SW. The remaining 25 samples were collected from inside the fenced area (Table 1). The sequence length was 720 bp including insertions and deletions, and the largest insertion of 77 bp as previously reported (Sorokin *et al.,* 2005) was found in two samples from the SW area. The large indels (insertion/deletion) sometimes influences in the result of analyses (Tajima, 1989). However, the calculated values were only slightly different with or without indels (data not shown), so each analysis below was carried out using sequences with indels.

We could not determine a clear relationship with respect to geographical location from the NJ phylogenetic trees (Fig.2). Some clades contained the samples from an area group, particularly in the Group 2, but totally, the samples obtained from an area group did not show cluster together in the tree. Of 103 samples nine pairs and a trio of samples shared identical sequences. Though sharing identical mitochondrial haplotype usually suggests the close matrilineal lineage or from a local group in the phylopatric species, the distances between the samples sharing a haplotype were not always large (mean: 154 km, range: 7–336 km) when calculated according to Bowring (1996).

Apart from the sampling locations, the two clear phylogenetic groups were detected as in previous study (Sorokin *et al.,* 2005). One genetic group contained larger number of samples (group 1) than the other (group 2). The branching was highly supported, and samples included in each of the two groups were fairly consistent also in trees constructed in other methods (data not shown).

The results of genetic diversity analyses are shown in Table 1. The overall haplotype diversity value reached 0.998, though nucleotide diversity (π) value was not high (0.050 +/- 0.024). The high haplotype diversity compared to nucleotide diversity lead to large negative value (Fs = -24.005, P = 0.003) in the Fu's test for selective neutrality, which is sensitive to past demographic expansion. *SSD* value in the mismatch distribution analysis was small and not statistically significant when calculated based on both of the demographic and spatial expansion models. The mismatch distribution was monomodal and fitted the model values well, particularly when calculated based on the population expansion model (Fig.3).

Discussion and Conclusion

In this study, we obtained a sufficient number of samples along the international railroad to

Table 1. Molecular indices for sampled gazelle population

Number of samples	*k*	*s*	*h* +/- *SD*	π +/- *SD*	Tajima's *D* (*P*)	Fu's *Fs* (*P*)	*SSD(d)* (*P*)	*SSD(s)* (*P*)
103	92	271	0.998 +/- 0.002	0.050 +/- 0.024	-0.287 (0.490)	-24.005* (0.003)	0.004 (0.634)	0.007 (0.380)

k, number of haplotypes; *s*, number of polymorphic sites; *h*, haplotype diversity (Nei, 1987); π, nucleotide diversity (Tajima, 1983); *SSD(d)*, sum of squared deviations from the observed and simulated mismatch based on the demographic expansion model; *SSD(s)*, sum of squared deviations from the observed and simulated mismatch based on the spatial expansion model. Asterisk indicates the statistical significance, $P < 0.05$ (The Fu's *Fs* was significant only when $P < 0.02$).
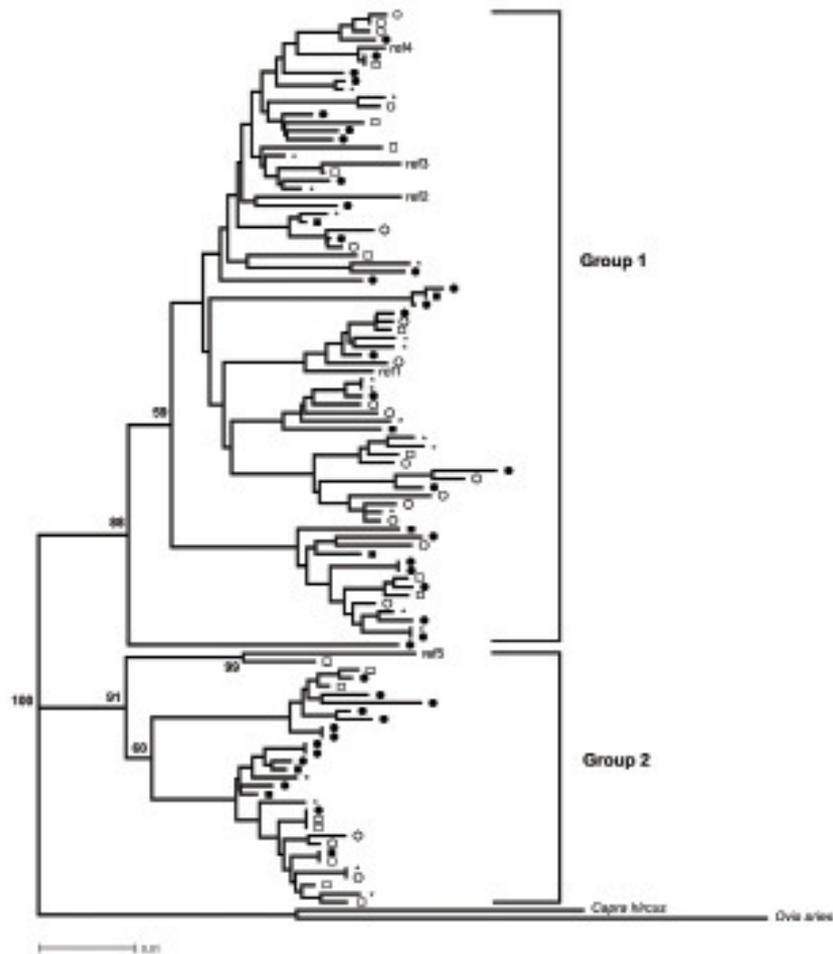
Figure 2. Phylogenetic NJ tree of the Mongolian gazelle samples, including those obtained in China and Russia as references. Symbols indicate the area from which the sample was obtained: ○NW, ●SW, □NE, ■SE, and *IN. Bootstrap values only at higher level nodes are shown. The accession numbers of the references are as follows: ref1 China (DQ266320), ref2 China (DQ266339), ref3 China (DQ266336), ref4 Russia (DQ266315), and ref5 Russia (DQ266305). The sequences of *Capra hircus* (DQ188898) and *Ovis aries* (DQ491618) were included as outgroup.
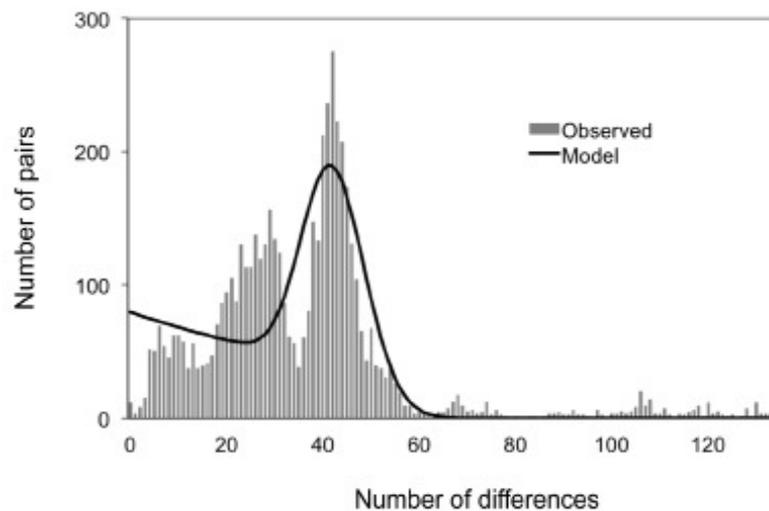

Figure 3. The frequency distribution of pair-wise differences of sequences for the sampled Mongolian gazelle population. The bars indicate observed frequencies and lines indicate expected frequencies based on the demographic expansion model.

analyze genetic diversity of gazelles. Gazelle carcasses with unknown cause of mortality were used for the genetic analysis. Despite the composition of sex and age structure of carcasses may differ from the gazelle population, we assume this potential discrepancy did not affect on our molecular results. Our results of the phylogenetic analysis showed that there are two genetic groups within the gazelle population, which confirms the previous research (Sorokin *et al.,* 2005; Sorokin & Kholodova, 2006). There is no clear geographical pattern in genetic structure and diversity of gazelles, although some clades contained samples from an area suggested slight spatial heterogeneity existed in the population.

Natural barriers to gazelle movements, such as a large river or seismic line, are absent in the study area; therefore, the railroad seems to be only barrier to the gazelle movement. In fact, Ito *et al.* (2005) observed that satellite-tracked gazelles did not cross the railroad. However, we did not find a clear genetic difference in the samples between western versus eastern part of the railroad even when including the isolation by distance effect of north versus south, implying the railroad is not working as a detectable barrier for gene flow at present. Presumably, the railroad is constructed too recently to form detectable genetic isolation in the large populations of gazelles. Additionally, during the survey we sometimes found carcasses inside of the fenced area, indicating the gazelles may cross the railroad occasionally. Highly variable marker such as microsatellites will be helpful to examine genetic structure in the population, which is used to detect the influence of the habitat fragmentation caused by artificial constructions such as railroads, roads and pipelines (e.g. Gerlach & Musolf, 2000; Riley *et al.,* 2006; Balkenhol & Waits, 2009; Lifeng *et al.,* 2011; Ji *et al.,* 2011). The markers may also provide us with the chance to examine to which extent the two genetic groups have segregated at present.

It is surprising the population showed large negative Fu's *FS* value, as such value is usually obtained when the population experienced population expansion, or in case the allele is linked to a gene with positive selection (Fu, 1997). The result of mismatch distribution analysis was not significant, which accepts the hypothesis of demographic expansion as well. The values in our mismatch distribution analysis

suggested the expansion did not occur recently, but may be tens of thousands years ago (data not shown). Rogers (1995) suggested initial demographic change often obscure the effect of later demographic change. Possibly, ancient population expansion of gazelles made it difficult to detect the known decline during 20th century. We believe it is necessary to investigate the demographic history further, carefully using both mitochondrial and microsatellite markers.

The nucleotide diversity in the sampled population was 0.050, which is similar extent determined for gazelle samples by a previous study (0.059; Sorokin *et al.,* 2005). When the genetic diversity of Mongolian gazelles was compared to those of other *Procapra* species, the value is larger than that of endangered *P. przewalskii* around Qinghai Lake in China (0.015; Lei *et al.,* 2003), but smaller than that of *P. picticaudata* in Tibet (0.081; Zhang & Jiang, 2006; Leslie, 2010), in spite that estimated population of *P. picticaudata* is much smaller (~ 100,000 individuals during 1990s; Leslie, 2010). Given infrastructure development is booming in Mongolia, there is a critical need to monitor the genetic diversity of the gazelle populations in relation to anthropogenic barriers, including railroad and highway. In addition, we urgently need to investigate the genetic structure and diversity of isolated small herds of gazelles in western Mongolia, where variation can be inexorably lost through genetic drift (Wright, 1969; Mills, 2007; Frankham *et al.*, 2002).

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