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# Genetic diversity of Przewalski's gazelle using noninvasive DNA and its implications for conservation

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**The Przewalski's gazelle (*Procapra przewalskii*) is endemic to China and the total current population of the species is only about several hundred. In order to understand the genetic structure and diversity of the Przewalski's gazelle in China for the purpose of guiding conservation initiatives, we examined the genetic variability and differentiation based on microsatellite loci, using noninvasive genetic sampling. 188 Przewalski's gazelle scats from six populations around Qinghai Lake (Tianjun, Haergai, Bird Island, Hudong-Ketu, Sand Island and Yuanzhe) were collected, and 128 individuals were identified and 94 alleles were detected at the 12 loci surveyed. The results showed that the expected heterozygosity and polymorphism information content is over 0.79 and 0.75, respectively. The AMOVA results showed that 5% of all observed diversity was due to difference between the populations and 95% was caused by genetic variance within population. According to the genetic similarity and geographical closeness, we suggested that six populations should be managed as three separate conservation units and habitat corridors should be built to link the Yuanzhe, Hudong-Ketu, Haergai and Sand Island populations.**

**Key words:** Habitat fragment, genetic diversity, noninvasive genetics, Przewalski's gazelle, *Procapra przewalskii*.

## INTRODUCTION

The Przewalski's gazelle is endemic to China and is the most endangered antelope species in the world (Jiang et al., 2001). According to results of a recent investigation, the population has declined to not more than ten populations near Qinghai Lake: seven populations clustered around the lake (Yuanzhe, Hudong-Ketu, Haergai, Talexuanguo, Ganzihe, Bird Island and Sand Island), one population in Tianjun County, and two populations in Gonghe County (Qiejitan and Wayu populations) (Ye et al., 2006). The total population size is estimated to be only about six hundred individuals (Ye et al., 2006). The critical status of this gazelle has aroused attention world-wide and it was listed as Critically Endangered by the Species Survival Commission of the International Union for the Conservation of Nature (IUCN) from 1996 to 2008, then as Endangered after 2008 (IUCN 2009). It has been a Category I (Endangered in China) National Protected Wild Animal Species in China since 1989 (Wang and Xie, 2004; Li, 2010). Since 1990s, some scientists have carried out several studies on the Prewalski's gazelle, including distribution and population size (Jiang et al., 2001; Ye et al., 2006), habitat selection and suitability

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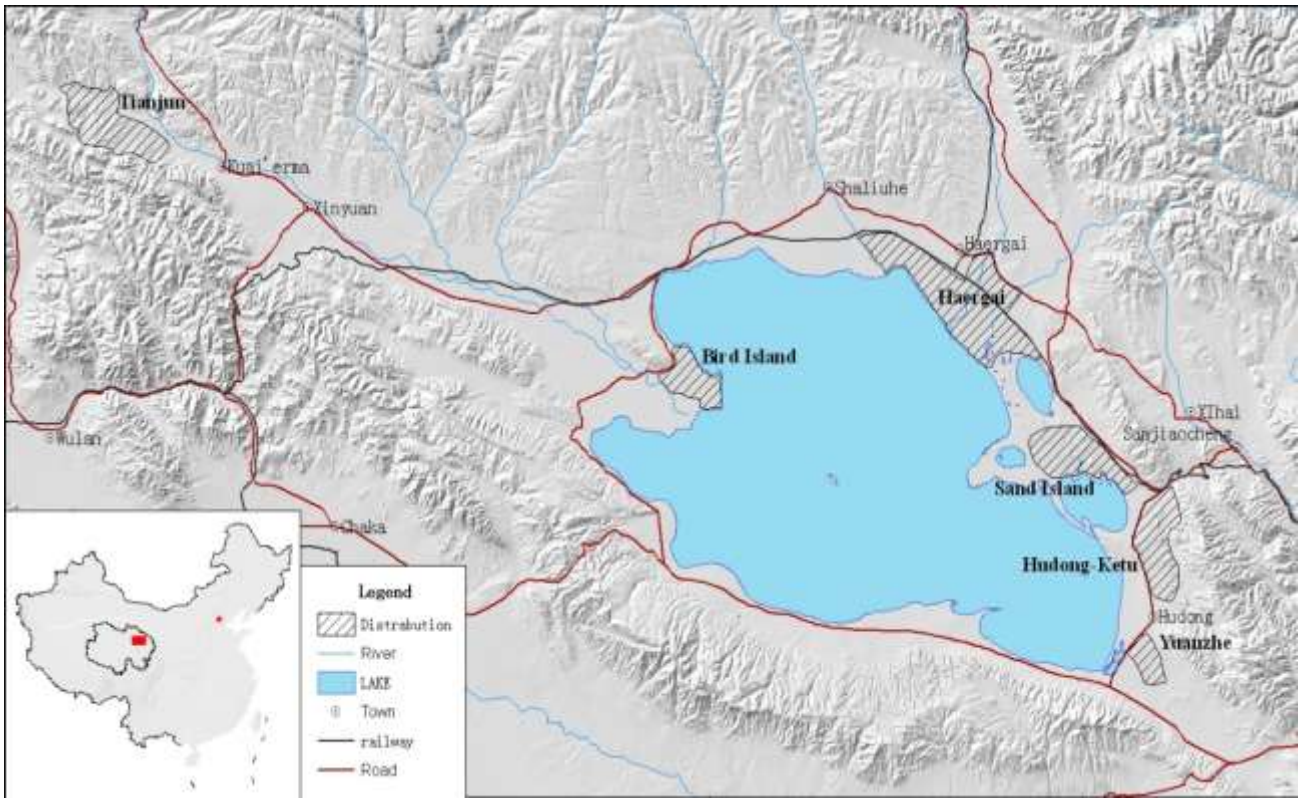


**Figure 1.** Map of sampling sites around Qinghai Lake regions in Qinghai province, China

(Liu et al., 2002a), food habits and foraging strategies (Liu et al., 2002b; Yi et al., 2005), courtship and mating behaviors (You et al., 2005), grouping patterns and social segregation (Lei et al., 2001), and genetic diversity and conservation strategies (Lei et al., 2003; Hu et al., 2010; Li et al., 2010; Yang et al., 2011). Determination of phylogeographic structure and information on distribution of genetic variation within and among populations is important for the purposes of conservation management, especially for the identification of evolutionary significant units (ESUs) and management units (MUs) for declining or endangered species, which can enable targeting of conservation efforts towards genetically distinct populations (Moritz, 1994). Molecular methods have become common for population monitoring, estimation of population parameters, and population viability analysis for rare and endangered species (Waits et al., 2005; Schwartz et al., 2007). Recently developed DNA extraction methods from noninvasive samples, such as hair, skin and scat, have enabled microsatellite loci analysis and greatly extended its applications in conservation of rare and endangered animals (Smith et al., 2006; Zhou et al., 2007). Microsatellites are currently one of the markers of choice for the molecular characterization of animal genetic diversity and conservation genetics.

In this study, we examined the genetic structure of six populations in the Qinghai Lake region and Tianjun using noninvasive genetic methods. This study is to understand the genetic structure and diversity of Przewalski's gazelle populations in China and their implications for conservation.

## METHODS

### Study area, sample collection and DNA extraction

This study was conducted at the six major distribution areas of Przewalski's gazelles around the Qinghai Lake (36°28'-38°25'N, 97°53'-101°13'E), Qinghai Province, China, including Bird island, Hudong-Ketu, Yuanzhe, Tianjun, Haergai and Sand Island (Figure 1). Qinghai Lake lies at 3,200 m above sea level and is 4,583 $m^2$. Qinghai Lake is fed by approximately 40 rivers and streams, but its water table has descended 3.7 m since 1959 due to long-term changes and drier climatic conditions (Ma et al., 2006). The area is characterized by a continental climate with dry and cold winters, strong winds in spring and winter, high levels of solar radiation, and a short frost-free period. The mean annual temperature is 1.1°C at Hudong on the east shore of the lake and 0.3°C at Jiangxigou on its south shore. Temperature extremes are 25 and -31°C. Annual precipitation varies from 360 to 370 mm in the north-west to 395 to 412 mm in the south-east, mostly between June and September (Li et al., 2010).

In this study, Przewalski's gazelle populations were noninvasively sampled by the collection of scats in six different populations around the Qinghai Lake and Tianjun (Figure 1). A total of 188 scats were collected from six study areas (Table 1). Samples were stored in 15 mL centrifuge tubes with about 12 mL of silica desiccant

**Table 1.** Primer sequences and annealing temperatures.

| Locus (accession no.) | Primer sequences (5'-3') | Annealing temperature (°C) |
|---|---|---|
| OArFCB304 | F: CCCTAGGAGCTTTCAATAAAGAATCGG<br>R: CGCTGCTGTCAACTGGGTCAGGG | 63 |
| SMHCC | F: ATCTGGTGGGCTACAGTCCATG<br>R: GCAATGCTTTCTAAATTCTGAGGAA | 58 |
| BM1862 | F: AAGCAAAAAGGCTGATGGC<br>R: TTGCAGTACTGGCAAGTGG | 58 |
| BM1392 | F: TTGTTTAGGCAAGTCCAAAGTC<br>R: AACACCGCAGCTTACTCC | 63 |
| TGLA54 | F: CTCAATATTTTGCAATAACATATAAGG<br>R: ACGATATCATGTTAGTTTCAGGTG | 63 |
| MNS64 | F: ATTAACTTTGTGGCATCTGAGC<br>R: CGTATCAACTAACACGATGCTG | 58 |
| MCM38 | F: TGGTGAATGGTGCTCTCATACCAG<br>R: ACGCCAGCAGCCTCTAAAGGAC | 58 |
| Oarae133 | F: AGCCAGTAGGCCCTCACCAGG<br>R: CCAACCATTGGCAGCGGGAGTGTGG | 63 |
| MNS61 | F: AAGTGTGGATCTGGATATTTGG<br>R: TCCTCTAAAACACCAACAATTTC | 58 |
| BM066 | F: ATCTGCCTGAAGCCAGTCAC<br>R: GGTTTCCTGCACCTGCATGA | 59 |
| BM1341 | F: CCTACCTACTGCACAGTTTTGC<br>R: CTCCCATATAAGTTACCCACCC | 59 |
| BM3501 | F: CCAACGGGTTAAAAGCACTG<br>R: TTCCTGTTCCTTCCTCATCTG | 58 |

covered by a clean Kimwipe tissue to separate the desiccant from the scat. DNA was extracted from scat using the Qiagen Stool DNA extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's recommendations.

#### Microsatellite loci PCR amplification

Twelve selected microsatellite loci were subjected to PCR amplification (Table 1). PCR amplification was carried out in a total volume of 25 µL reaction mixture containing 1× PCR buffer, 1 µl DNA as template, with each dNTPs at a concentration of 200 µmol/L, 2.0 mmol/L $Mg^{2+}$, each primer at a concentration of 1 µmol/L, 4 µg of bovine serum albumin (BSA) and 1.0 U of Taq DNA polymerase. The cycling profile included an initial denaturation step at 95°C for 2 min, followed by 50 cycles of 30 s at 94°C, 30 s at 58 to 63°C depending on the primer pair used (Table 1), 1 min at 72°C, and a final step of 5 min at 72°C using a GeneAmp 9700 (Applied Biosystems) thermal cycler. To avoid potential sample deviation and minimize PCR artifacts, three replicate amplifications were carried out for each sample.

#### Microsatellite genotyping

The PCR fragments were fractionated and sized using an automated ABI377 DNA sequencer and the internal size standard Genescan 350 TAMRA (Applied Biosystems). Data were collected with ABI PRISM 377 software (Applied Biosystems). Fluorescent DNA fragments were analyzed using the GENESCAN (version 3.1) and the GENOTYPER (Version 2.0) software (Applied Biosystems) (Mburu et al., 2001). Samples that did not yield two or more consistent genotypes were discarded from the analysis.

#### Data analysis

The probability of identity ($P_{ID}$) and siblings ($P_{ID}$-sibs) were estimated in GENEALEX (Version 6.0) for unrelated individuals by microsatellite (Peakall and Smouse, 2006). Estimates of genetic diversity including number of alleles (A), effective number of alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He) and Wright's F-statistics ($F_{ST}$) were calculated using GENEALEX (Peakall and Smouse, 2006). Polymorphism information content (PIC) was calculated using CERVUS 3.0.3. To quantify the population structure within and between the analyzed genetic groups of Przewalski's gazelles, the Analysis of Molecular Variance (AMOVA) was obtained using GENEALEX software. Genetic divergence between populations based on allele frequencies was calculated according to Nei's unbiased genetic distance using GENEALEX, and UPGMA dendrogram of six populations was constructed using genetic distance.

## RESULTS

### Genotyping analysis and individual identification

Of the 188 scat samples, 133 had successful amplification at all 12 microsatellite loci, and the success

**Table 2.** Scats collected during field survey in six study areas and genotyping result by microsatellites.

| Study areas | Tianjun | Bird Island | Haergai | Sand Island | Yuanzhe | Hudong-Ketu |
|---|---|---|---|---|---|---|
| Scats | 32 | 30 | 54 | 24 | 22 | 26 |
| Complete microsatellite genotypes | 21 (65.63%) | 21 (70.00%) | 43 (79.63%) | 15 (62.50%) | 16 (72.73%) | 17 (65.38%) |
| $P_{ID}$ | $4.66\times10^{-15}$ | $1.02\times10^{-14}$ | $5.51\times10^{-15}$ | $1.70\times10^{-15}$ | $2.92\times10^{-17}$ | $1.56\times10^{-16}$ |
| $P_{ID}$-sibs | $5.97\times10^{-6}$ | $6.85\times10^{-6}$ | $2.56\times10^{-6}$ | $4.63\times10^{-6}$ | $2.28\times10^{-6}$ | $3.02\times10^{-6}$ |

**Table 3.** Genetic variability at 12 microsatellite loci in 6 populations.

| Parameter | Tianjun | Bird Island | Haergai | Sand Island | Yuanzhe | Hudong-Ketu |
|---|---|---|---|---|---|---|
| The number of effective allele | 5.36±0.47 | 5.25±0.51 | 6.52±0.46 | 5.52±0.33 | 6.65±0.40 | 6.14±0.35 |
| Observed heterozygosity | 0.901±0.020 | 0.898±0.018 | 0.884±0.023 | 0.910±0.023 | 0.923±0.021 | 0.925±0.018 |
| Expected heterozygosity | 0.798±0.016 | 0.793±0.016 | 0.839±0.011 | 0.811±0.012 | 0.844±0.009 | 0.831±0.010 |
| polymorphism information content | 0.785±0.016 | 0.759±0.020 | 0.817±0.013 | 0.791±0.014 | 0.821±0.001 | 0.815±0.012 |

**Table 4.** F-statistics for the 12 loci in all six populations.

| Study Population | Tianjun | Bird Island | Haergai | Sand Island | Yuanzhe |
|---|---|---|---|---|---|
| Bird Island | 0.052 | | | | |
| Haergai | 0.022 | 0.027 | | | |
| Sand Island | 0.035 | 0.041 | 0.021 | | |
| Yuanzhe | 0.019 | 0.026 | 0.010 | 0.025 | |
| Hudong-Ketu | 0.026 | 0.028 | 0.012 | 0.028 | 0.011 |

rate for complete genotypes was 62.50 to 79.63% in six study populations (Table 2). PID and PID-sibs analysis results showed that the 12 loci were sufficient for distinguishing individuals in the six populations. Overall, we identified 128 unique genotypes that represented 128 Prezewlaski's gazelle individuals.

## Within breed diversity

94 alleles were detected at the 12 loci surveyed among the 128 individuals. For all samples, loci BM1341 had the highest allele number (n = 11), and loci BM 1329 and BM1862 had the lowest allele numbers (n = 6). The average number of alleles per locus was 7.83. The number of effective alleles was 5.25 to 6.65 in the six populations (Table 3). The Yuanzhe population and Bird Island population had the highest and lowest number of effective allele, respectively.

Ho, He and PIC were calculated for the six populations (Table 3). As shown in Table 2, He was lower than Ho in all populations. Ho for Hudong-Ketu population was the highest (0.925) and for Haergai population, the lowest (0.884), however, He for Yuanzhe population was the highest (0.844) and for Bird island population, the lowest. Microsatellite loci in different populations showed differences in polymorphism and calculated PIC. PIC for the six populations were all over 0.50 with Yuanzhe population, the highest (0.821), showing that these populations were highly genetic and diversed.

## Genetic differentiation between populations

In accordance with the analysis of existing genetic differentiation between the possible pairs of genetic groups (Table 4), the Fst values showed a higher differentiation ($P<0.01$) between the Tianjun and Bird Island populations, being the highest (0.052), and between the Yuanzhe and Sand Island population (0.010), the lowest. The AMOVA results showed that 5% ($P = 0.053$) of all observed diversity was due to difference between the population and 95% resulted to the individuals within population.

Genetic divergence was calculated using GENEALEX and UPGMA dendrogram (Table 5). The largest genetic distance (0.544) existed between the Tianjun and Bird Island populations, and the lowest genetic distance

**Table 5.** Nei's genetic distance between populations in all 6 populations.

| Study population | Tianjun | Bird Island | Haergai | Sand Island | Yuanzhe |
|---|---|---|---|---|---|
| Bird Island | 0.544 | | | | |
| Haergai | 0.213 | 0.281 | | | |
| Sand Island | 0.353 | 0.427 | 0.231 | | |
| Yuanzhe | 0.187 | 0.268 | 0.108 | 0.283 | |
| Hudong-Ketu | 0.263 | 0.284 | 0.132 | 0.314 | 0.116 |

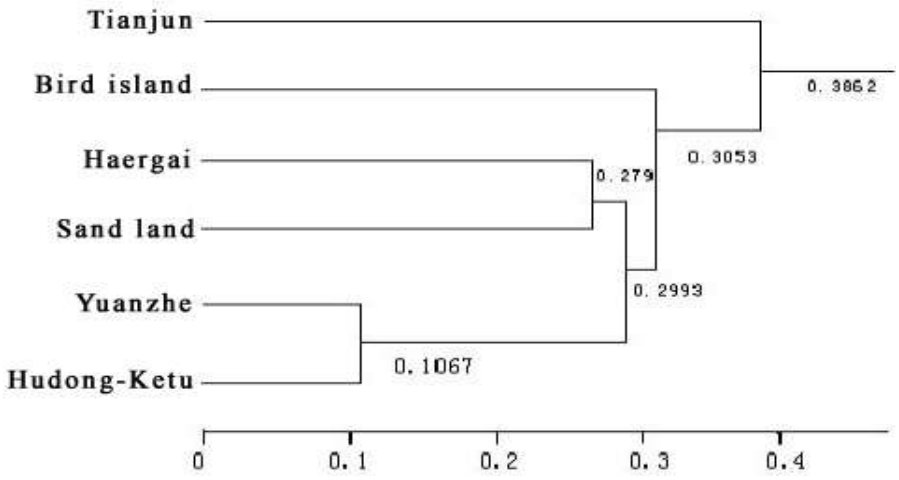


**Figure 2.** UPGMA dendrogram of six populations using genetic distance.

(0.116) was between the Yuanzhe and Hudong-Ketu populations. The UPGMA dendrogram of six populations was constructed using genetic distance (Figure 2). The dendrogram indicated a separation into four distinct groups. The first cluster was formed by the Yuanzhe population and the Hudong-Ketu population. The second cluster was formed by the Haergai and Sand Island populations, and the Bird Island and Tianjun populations were two isolated clusters.

## DISCUSSION

This is the first attempt to specifically quantify genetic diversity of most of the Przewalski's gazelles populations with microsatellite markers. To understand better the effect of habitat fragmentation and geographical isolation on gene flow and genetic variation, and uncover genetic units for conservation, Lei et al. (2003) examined the hypervariable region of the mitochondrial DNA control region from Bird Island, Hudong-Ketu, Yuanzhe and Sand Island populations, and the results showed that the nucleotide diversity within population was very low (less than 0.004) and gene flow between populations was low. In this study, the results showed that six Przewalski's gazelle populations had high level of genetic heterozygosity and high polymorphism information content in these 12 microsatellite loci than some other endangered and rare animal populations (Evdotchenko et al., 2003; Zhou et al., 2007) (Figures 3 and 4). There are two reasons for the high genetic diversity for these populations. Firstly, we selected and used the high polymorphic microsatellite DNA as the genetic markers. The detected mean allele of microsatellite is 7.83, therefore, the microsatellites used in this study were suitable for genetic diversity analysis. Secondly, the analyzed samples were from six populations which covered more population size.

Conservation genetics can provide information contributing to conservation planning, and the genetic structure of populations has crucial implications for management and conservation (Mockford et al., 2005). To maintain biological diversity within Przewalski's gazelle populations around Qinghai Lake, it is essential for this remnant genetic variation to be preserved both within and among extant populations (Lei et al., 2003). As a consequence of human activity, habitat loss and illegal hunting, the Prezewalski's gazelle declined dramatically and divided into several populations and their habitat were fragmented into different isolated patches (Jiang et al., 2001). The Fst and Nei genetic distance analysis showed that the Tianjun population has the highest genetic variance and genetic distance with Bird Island population,

**Figure 3.** A running Przewalski's gazelle (photo by Yonglin Wu).

**Figure 4.** A Przewalski's gazelle population living in fenced paddocks (photo by Yonglin Wu).

however, the Hudong-Ketu and Yuanzhe populations had the lowest genetic variance and genetic distance. These studies revealed a marked divergence of lineages of the Tianjun and Bird Island populations from the other populations, which corresponds to the geographical association. Furthermore, Hudong-Ketu, Yuanzhe, Haergai and Sand Island populations are close to each other geographically and genetically (Figures 1 and 2). Therefore, we suggested that the Yuanzhe, Hudong-Ketu, Haergai and Sand Island populations should be managed as one conservation unit, and the Tianjun and Bird Island also should be managed as two separate conservation units.

The human activities are still threatening the Przewalski's gazelle conservation and genetic management. Although, the Przewalski's gazelle is one of the main target species of protection in the Qinghai Lake Nature Reserve which was set up in 1997, only gazelles on the Bird Island are within the protected area (Jiang, 2004). To improve livestock productivity, grasslands were leased to local herdsmen who have transformed them into fenced paddocks (Liu et al., 2002b), and the policy will continue for 50 years. Usually, the fence lines around Qinghai lake are about 1.2 to 1.5 m and have 9 lines, especially the top line with thorns. Double fence lines were built in Haibei state for different management department and projects. They not only induced the possibility of gazelle to escape from wolf, but made gazelle to die, get injured and disabled. According to the patrol records of Qinghai Lake Nature Reserve, the total of 120 gazelles dead was because of fence lines from 2001 to 2011. These fence lines are one main reason for the declined population. Meanwhile, these fence lines impair the ability of gazelle individuals to migrate within and among populations. Road networks around the lake also have formed barriers to the free movement of gazelle (Li et al., 2009). These barriers may reduce the gene flow within or among populations and increase the risk of inbreeding. The genetic diversity of each popula-tion is not a conservation aim, rather the genetic diversity of the entire species should be ensured. Therefore, we should set up communications between (sub)-populations to allow gene flow between population. Therefore, we suggested that the game fences on rangelands should be removed and habitat corridors should be built to link the Yuanzhe, Hudong-Ketu, Haergai and Sand Island popula-tions.

## Conflict of interest

Authors did not declare any conflict of interest.

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