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Full Length Article

The Genetic Diversity of Tibetan Red Deer Populations Determined using Mitochondrial and Microsatellite Markers

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Abstract

The Tibetan red deer (*Cervus elaphus wallichi*) is an indigenous Chinese species, mainly inhabiting Sangri County, located in the southeast segment of the Tibetan Autonomous Region. Currently, scientific data on the Tibetan red deer are sparse. Knowledge of the genetic diversity of geographically separate populations could help in formulating strategies to protect this species. In this study, we collected 123 fecal samples from three different regions. These contained 105 samples identified as red deer feces from which 54 genotypes were obtained and analyzed, including 21 Zengqi, 15 Woka and 18 Baidui populations. We performed genetic analysis using mitochondrial and microsatellite markers. The mtDNA CytB genes from 54 individual Tibetan red deer were sequenced and 731 bp fragments were obtained. We defined 14 haplotypes with a haplotype diversity (*H*) of 0.897. The nucleotide diversity (π) between populations was 2.781. Microsatellite typing analysis showed that the average allele number, mean expected heterozygosity and mean observed heterozygosity were 4.385, 0.721 and 0.641, respectively. Our findings suggest that these Tibetan red deer populations have relatively high genetic diversity. As indicated by Tajima′s *D* and Fu and Li′s *D* values, these three populations had not significantly deviated from a neutral evolutionary path ($p > 0.1$), nor was any evidence found to suggest a strong equilibrium selection. AMOVA revealed that the inter-gene flow of these three populations was greater than 1 ($5.14 > Nm > 1.67$), suggesting that abundant gene flow exists between them. No obvious genetic differentiation was observed. Thus, we propose to protect and control the Tibetan red deer in these three regions as a single management unit.

Keywords: Tibetan red deer; CytB; Microsatellite; Genetic diversity

Introduction

The red deer (*Cervus elaphus*) was once widely distributed throughout the palaearctic and nearctic regions and based on coat colors and morphological characteristics of skulls and antlers has been classified into 22 subspecies (Flerov, 1952; Ohtaishi, 1995). The Tibetan red deer (*Cervus elaphus wallichi*), which is of medium size is one such subspecies (Sheng and Ohtaishi, 1993).

Historically, *C. e. wallichi* has been distributed throughout Tibet, Sikkim, Nepal and Bhutan (Fig. 1) (Bailey, 1911; Ludlow, 1959). However, there have been few published studies of the subspecies and these have reported only quantitative statistics and geographic distribution data (Caughley, 1970; Feng *et al.*, 1986). In 1959, Ludlow reported the activities of wild Tibetan red deer (Ludlow, 1959). Following this, there were no reports for several decades, until Thornback (1978) announced that the Tibetan red deer might become extinct, while Dolan and Killmar (1988) regarded this as a fictional animal. World Wildlife Fund (personal communication) announced in 1992 that the Tibetan red deer had become extinct in the wild. Nearly 20 years after its last sighting, in year 2005 the subspecies reappeared in the valley of the middle reaches of the Yarlung Zangbo River (Fig. 1; personal communication). In order to further understand how Tibetan red deer adapt to environmental changes (Bauert *et al.*, 1998) and to stabilize the fluctuating population and maintain the dynamic balance of the ecosystem (Yachi and Loreau, 1999; Hughes *et al.*, 2008) it is necessary to investigate the genetic basis of the population in the wild.

The aim of this research was to contribute to a more complete molecular analysis of Tibetan red deer populations to increase our knowledge of the subspecies. To do this, we obtained red deer fecal samples from a distributional area in western China and obtained molecular information from mitochondrial sequences and microsatellite markers. The mitochondrial genome the cytochrome b gene has been used frequently in species identification and in research of genetic structure and diversity in the deer population (Hartl *et al.*, 2003; Ludt *et al.*, 2004; Wu *et al.*, 2004; Hartl *et al.*, 2005) because it is maternally inherited and has a relatively rapid mutation rate (Wu *et al.*, 2004). The microsatellite marker has been an effective means of identifying individuals and investigating genetic variation and population structure in wildlife conservation and

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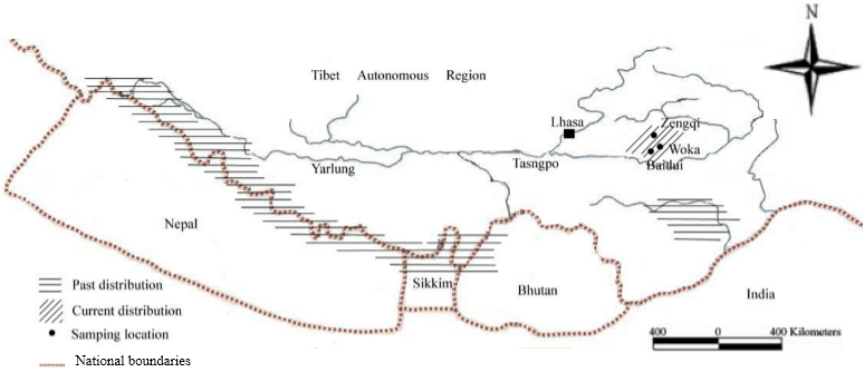

Fig. 1: Past and current distribution of Tibet red deer and sampling locations in this research

management (Broders *et al.*, 1999; Polziehn *et al.*, 2000) because it is bi-parentally inherited and is abundant across the genome, highly polymorphic and allows for easy genotyping (Røed, 1998). In this study, we used both the cytochrome b gene and microsatellite loci as markers to assess the level of genetic diversity and differentiation of three red deer populations from Zengqi, Woka and Baidui. We also examined the implications of the obtained information for establishing management and conservation strategies for red deer in Tibet and western China.

Materials and Methods

Sample Collection and DNA Extraction

Fecal samples (n = 123), as fresh as possible, were collected during direct, telescopic, behavioral observations and were stored in test tubes containing 95% ethanol (Tibetan red deer excrement is 2–3 cm in length, 1–2 cm in width, either bullet-or jujube pit-shaped). In order to prevent contamination from other samples, each pellet was labeled and we changed gloves between each sample collection.

Before conducting DNA extraction and PCR amplification on the fecal samples we selected those with the highest utilization rate based on external observation to enhance the efficiency of the process. Samples with shallow surface colors possess relatively little pigment and the quality and quantity of DNA extracted are higher than from those samples with deep surface color. For Tibetan red deer, the optimal method for extracting DNA is to use the outermost layer of the fecal pellets, which seems to maximize DNA yield and minimize inhibitor content (Wehausen *et al.*, 2004). The fecal DNA extraction process used the DNA Stool Mini Kit by QIAGEN (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The detailed steps are as follows: the red deer feces of 1 mL was put into the conical centrifuge tube and some buffer solution was added. Solid matter was fully dissolved through vortex oscillation after, which the tube was centrifugated and contaminants were precipitated. The supernatant in the tube was then aspirated and placed into another conical tube and the enzymatic hydrolysate of 2 mL, salting-out liquid and interference removing agent were added. After vortex oscillation the mixture was placed in an incubator at 60℃ to lyse the cells. After lysis was complete, the extract was added to the solution. After oscillation and centrifugation the supernatant was injected into another conical centrifuge tube. After adding 2 mL of concentrate and precipitation solution, the centrifuge was again used to remove the supernatant and after adding the cleaning fluid, the centrifuge was used and the supernatant discarded, once more. After the substance remaining in the tube had dried naturally in the air, the preserving fluid was added and the mixture was placed in a refrigerator at 4℃ before analysis. To prevent contamination during DNA extraction, benches, pipettes and laboratory equipment were wiped down daily and after each batch of samples was processed. Researchers also changed gloves between taking samples to avoid cross-contamination.

Species Identification

Species identification was performed utilizing a region of the mitochondrial cytochrome b gene with the primers A1 (5'-GAAAAACCATCGTTGTCATTCA-3') and B2 (5'-GGAGGTTGG AGCTCTCCTTTT-3') (Christian *et al.*, 2004). Polymerase chain reactions (PCRs) were carried out in a final volume of 50 µL containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM Mg^{2+}, 200 µmol of each dNTP, 1.5 U Hotstar Taq DNA polymerase (QIAGEN), 1 µg $µL^{-1}$ BSA, 0.2 µmol of each primer and ≤ 10 ng of genomic DNA.

The reaction process was as follows: an initial pre-denaturing at 95°C for 15 min, denaturing at 95°C for 60 s, annealing at 56°C for 60 s, primer extension at 72°C for 60 s and a final extension at 72°C for 10 min. Products were visualized on 2% agarose gel stained with GelRed (Biotium), purified using Qiagen PCR Purification Kits (Qiagen) and detected on an ABI 3730 DNA sequencer. During the experiment, DNA extracted from red deer muscle and deionized water were used as positive and negative controls, respectively. Multiple amplification and bidirectional sequencing were used simultaneously to

reduce possible errors. Finally, the most similar sample sequences were compared using Megablast on the NCBI website.

Individual Identification

Referring to published literature, we selected 11 pairs of microsatellite primers with high polymorphism and stable amplification, which were used for the identification of individuals and population genetic analysis (except BM848, BM203 and OCAM). The selected microsatellite loci were CSSM19 (Kuehn *et al.*, 2003), N, BM848 and BM203 (Bishop *et al.*, 1994), OCAM (Moore *et al.*, 1992), T507, T123, T156, T530, T501 (Jones *et al.*, 2000) and C143 (Meredith *et al.*, 2005). FAM, HEX, or TAMRA was used to label the primers by fluorescence (Sangon Biotech (Shanghai) CO., Ltd). Microsatellite primer sets were run either in multiplexed groups or individually. A high temperature start of 95°C for 1 min was used to initiate reactions for each unit of Taq DNA polymerase. During the reaction, PCR thermocycling was performed at 94°C denaturation for 15 s, 54–57°C annealing for 30 s and 72°C extension for 45 s for 45–52 cycles, followed by a final extension step at 72°C for 15 min. During this PCR reaction, both negative and positive control reactions were performed simultaneously. The positive control was a known microsatellite genotype from red deer muscle samples and the negative control was sterile water and PCR reagents. The PCR products with a fluorescent-labeled base pair size standard (ROX-350, Applied Bio systems) in each lane were electrophoresed on 5% acrylamide 7 M urea gels using an ABI 3730 DNA sequencer. Each sample was subjected to at least three PCR reactions to improve the accuracy of the experimental results (Koban *et al.*, 2011). We used GeneScan 672 to perform image analysis and Genotyper software (Applied Biosystems) for fragment size determination. Genotype was divided into homozygote and heterozygote, with two observed DNA bands indicating heterozygosity and one band indicating homozygosity (Ernest *et al.*, 2000).

Population Genetic Analysis

Mitochondrial DNA sequences were visually confirmed and aligned by the CLUSTAL_X program (Thompson *et al*., 1997), which also identified haplotypes. Pairwise sequence differences between haplotypes were calculated using MEGA 3 (Kumar *et al.*, 2001). Genetic diversity within populations was estimated by haplotype (h) and nucleotide (π) diversities using DNAsp 3.0 (Rozas and Rozas, 1999). Haplotype diversity is the probability that two randomly chosen individuals in a population have a different haplotype. The number of differences between individual haplotypes was not considered; nucleotide diversity was considered for the number of different haplotypes and for their degree of similarity. The sequence results obtained from mitochondrial DNA were arranged by CLUSTAL_X, with manual proofreading.

The DNAsp 4.0 software package was used to calculate nucleotide (π) and haplotype diversity (h) (Rozas and Rozas, 1999). The MEGA 2.1 program was used for sequence comparison and variation detection to determine the variation sites and haplotypes (Kumar *et al.*, 2001).

Additionally, PAUP 4.0b10 and MrBayes 3.0 softwares were used in the construction of phylogenetic trees through calculation of maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) (Swofford, 2002; Ronquist and Huelsenbeck, 2003). The most appropriate DNA substitution model and relevant parameters were tested and searched using MODELTEST 3.06. The confidence values for the branches of the phylogenetic trees were obtained by bootstrap (the number of repetitions being 1000) (Posada and Crandall, 1998). The *Capreolus capreolus* sequence (AY580069) was adopted as the outgroup for determining the tree roots in the construction of phylogenetic trees. Bootstrap analyses were performed with 5000 replicates for MP and 100 full heuristic replicates for ML. We used the MRBAYES program (version 3.0) (Huelsenbeck and Ronquist, 2001) for Bayesian phylogenetic inference. We ran four Markov chain Monte Carlos (MCMC) for 100,000 generations, sampling every tenth generation; the initial 5% of trees were discarded.

For the microsatellite genotype data, we used GIMLET v 1.3.3 software (Valiere, 2015) to calculate the probability of identity (PID) and the probability of identity among siblings (PID-sibs). Unique multilocus microsatellite genotypes were identified using the identity analysis module of CERVUS (Marshall *et al*., 1998). GENEPOP 3.4 was used to carry out all standard population genetic analyses. Using the Markov chain method, heterozygote deficiency was tested and compared to the Hardy-Weinberg equilibrium for each locus and globally. Additionally, we calculated the number of alleles per locus (*A*), Nei's unbiased expected heterozygosity under Hardy-Weinberg equilibrium (*He*) and the observed heterozygosity (*Ho*). Hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) was performed to compare levels of genetic diversity within and among several population groupings of red deer using ARLEQUIN 3.0 (Schneider *et al.*, 2000) with 1000 permutations.

Results

Species and Individual Identification

Fecal samples from 123 deer in the distribution area were collected and the cytochrome b gene sequences from the samples' mitochondrial DNA (mtDNA) were analyzed. We used BLAST (Megablast) at NCBI to identify 105 sample sequences that had the least difference (< 3%) and the greatest similarity (> 97%) to the published cytB sequence,

AY04486 of the Tibetan red deer. We performed genotype analysis of these samples using 11 microsatellites (Table 1) and we obtained 54 different genotypes. The analysis using CERVUS 2.0 software suggested that the combined distinguish rate of microsatellite loci was high. Even in the case of identical twins the error probability, Psib, was only 0.12%. In nature, the probability of the fully consistent genotypes between two different individuals is 10^{-14}. In forensic science, fully consistent genotypes in two samples indicate the same individual or identical twins. Based on the above criteria, we identified 54 different individuals through behavioral observation and molecular analysis of fecal samples obtained from 105 Tibetan red deer.

Populations' Genetic Diversity

Regions of mtDNA cytB, with a length of 731 bp, were obtained from 54 individual Tibetan red deer. We defined 14 haplotypes using GenBank (Accession numbers FJ611888–FJ611891, the rest were submissions). In total, 24 mutations were identified, all of which were site mutations and no insertion or deletion was detected. Sequencing analysis revealed that the Baidui population had low levels of haplotype and nucleotide diversity ($h = 0.592 \pm 0.129$, $\pi = 0.546 \pm 0.00273$). In contrast, the Zengqi population showed high levels of haplotype diversity ($h = 0.807 \pm 0.024$) and nucleotide diversity ($\pi = 2.490 \pm 0.02014$). The overall haplotype and nucleotide diversities of the study population were 0.897 ± 0.014 and 2.781 ± 0.02465, respectively (Table 2). Tajima′s D and Fu and Li′s D test results indicate that the divergence of these three Tibetan red deer populations relative to neutral evolution showed no significant deviation ($p > 0.1$).

Table 3 lists the microsatellite genetic parameters of the three populations. The average value of expected heterozygosity (*He*) for each population ranged from 0.708 to 0.736. The average values of observed heterozygosity (*Ho*) varied between 0.583 and 0.655. The Zengqi population had low levels of effective allele numbers, observed heterozygosity and expected heterozygosity. The levels of these features were all higher in the Baidui population. The eleven microsatellite loci deviated from the Hardy-Weinberg equilibrium.

Populations' Genetic Structure

The constructed ML, MP and BI trees displayed a similar structure. The samples from different regions did not form significant geographical clusters (Fig. 2). Among the 14 haplotypes, HT2 and HT6 were shared by Zengqi and Woka, HT1 was shared by Zengqi and Baidui and HT8 was shared by Woka and Baidui. AMOVA software was used to detect genetic parameters in these three populations. The results show that the F*st* value between the Zengqi and Baidui populations was 0.21 and the gene flow ($Nm = (1 / Fst - 1)/2$) between them was 2.45 ($\Phi st = 0.274$, $p > 0.05$).

Table 1: Genetic information regarding eleven microsatellite loci in three red deer populations

loci	*A*	*Ne*	*Ho*	*He*	*P-val*	*Fis*	*PIC*	P_{ID}	*Psib*
CSSM19	10	3.502	0.534	0.624	0.1214	0.0945	0.517	0.068	0.370
BM848	8	4.765	0.715	0.614	0.3104	0.1247	0.264	0.064	0.366
T123	9	4.315	0.624	0.718	0.0459*	0.0347	0.519	0.070	0.458
T156	9	2.648	0.584	0.761	0.2657	0.0264	0.617	0.081	0.324
N	7	3.781	0.617	0.617	0.1257	-0.0064	0.607	0.076	0.509
BM203	10	5.624	0.614	0.815	0.0014**	0.3247	0.716	0.109	0.501
OCAM	5	4.915	0.654	0.864	0.0031**	0.0416	0.518	0.094	0.451
T507	6	5.459	0.513	0.614	0.0831	0.0461	0.614	0.261	0.376
T530	7	3.647	0.715	0.715	0.0007**	0.0517	0.719	0.101	0.423
T501	5	4.516	0.614	0.815	0.0967	-0.3014	0.764	0.074	0.491
C143	12	4.158	0.571	0.764	0.0715	0.2354	0.694	0.096	0.402
Mean	8	4.385	0.614	0.720	0.1297	0.0894	0.595	——	——

A: Number of alleles; Ne: effective number of alleles; Ho: observed heterozygosity; He: expected heterozygosity; P-val: site of Hardy-Weinberg balance test probability value; Fis: inbreeding coefficient in groups; PIC: polymorphism information content; PID: non-related idiotype similar probability; Psib: probability of identity among siblings. **p<0.01; *0.05 > p > 0.01

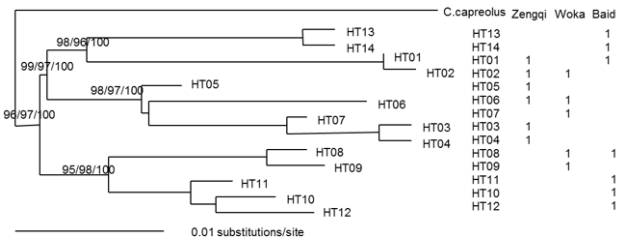

Fig. 2: Maximum-likelihood, maximum parsimony and Bayesian methods tree for all 14 haplotypes of *Cervus elaphus wallichi* and *Capreolus capreolus* (Z70318), which was used as an outgroup. Values above branches indicate support for each node based on maximum likelihood/maximum parsimony/Bayesian inference. Bootstrap values below 90% are not shown. Single maximum likelihood tree (-ln likelihood = 1273.8), estimated under the GTR + I + G model of substitution (with a gamma-shape correction of 1.3649, nucleotide frequencies: A = 0.3297, C = 0.2229, G = 0.1463, T = 0.3011 and an assumed proportion of invariable sites of 0.7488). HT: haplotype

The F*st* value between the Zengqi and Woka populations was 0.14 (Nm = 1.67; $\Phi st = 0.385$, $p > 0.05$). The F*st* value between the Woka and Baidui populations was 0.29 (Nm = 5.14; $\Phi st = 0.404$, $p > 0.05$) (Table 4).

Discussion

Genetic diversity is an essential factor that determines the ability of a species to evolve and therefore, survive (Frankham *et al.*, 2002). It is one of the important scientific bases for developing effective strategies to protect and manage rare and endangered species. Loss of genetic diversity has direct disadvantageous effects on species' survivability (David, 1998), making the species vulnerable to extinction (Hedrick *et al.*, 1995).

Table 2: Analysis of the genetic diversity of mitochondrial DNA cytB for 54 individual Tibetan red deer from three sampling locations

PN	NSI	SIS	CMG	NH	*h*	π	Tajima′s D	Fu and Li′s D
Zengqi	40	32	18	6	0.807±0.024	2.490±0.02014	0.19626*	1.43761*
Woka	40	35	15	5	0.742±0.034	2.067±0.02391	-1.19130*	-1.46075*
Baidui	43	38	21	7	0.592±0.129	0.546±0.00273	-1.06058*	-1.04443*
Total	123	105	54	14	0.897±0.014	2.781±0.02465	0.59276*	0.14348*

PN: population name, NSI: number of sampled individuals, SIS: species identification scat, CMG: complete microsatellite genotypes, NH: number of haplotypes, *h*: haplotype diversity, π: nucleotide diversity. *p > 0.1

Table 3: Analysis of the genetic diversity of 11 SSR loci in three populations of red deer

Population	Sample size	Ne	Ho	He	HWE
Zengqi populations	18	3	0.583	0.708	0.183
Woka populations	15	4	0.604	0.716	0.164*
Baidui populations	21	5	0.655	0.736	0.194

Ne: effective number of alleles, He: observed heterozygosity, HWE: Hardy-Weinberg equilibrium. *population deviated from Hardy-Weinberg equilibrium

Table 4: Genetic differentiation index between three subpopulations

Population	Zengqi populations	Woka populations	Baidui populations
Zengqi populations	——	1.67	2.45
Woka populations	0.14+	——	5.14
Baidui populations	0.21+	0.29+	——

Note: Nm (above diagonal), Fst (below diagonal), +P > 0.1, not a significant difference

The increased genetic similarity and decreased variation within a population cause reduced genetic diversity, which is common in many rare and endangered species (Frankham, 1995). Genetic analysis in the present study reveals that the Tibetan red deer has higher genetic diversity (*He* = 0.721) than other cervid species. For example, the expected microsatellite heterozygosities of moose (*Alces alces*), reindeer (*Rangifer tarandus*) and sika deer (*Cervus nippon*) are 0.325, 0.364 and 0.365, respectively (Broders *et al*., 1999; Cote *et al.*, 2002; Goodman *et al.*, 2001). Mitochondrial DNA is the only observed extranuclear genetic substance in animals and is acquired through maternal inheritance. Its structure is simple and stable, there is no gene recombination between generations and the evolutionary rate is fast. Nuclear genes can respond to genetic information within the nucleus and can provide useful information for studying genetic variance in animals. The utility of these two types of markers in the investigation of genetic evolution has been widely documented and proven in the literature (Avise *et al.*, 1987). The present research found the genetic diversity of Tibetan red deer to be 2.78%, which was higher than for some other species. For example, the nucleotide diversity of roan antelope (*Hippotragus equines* Saint-Hilaire) is 1.90% (Alpers *et al*., 2004), of English red deer (*Cervus elaphus* L.) is 0.56% (Hmwe *et al*., 2006) and of Chinese sika deer is 2.11% (*Cervus Nippon*) (Liu *et al*., 2003). Geomorphologic analysis reveals that the Gangdise Mountain region studied in this paper was formed by the collision, extrusion and fold rise of the Indian and Asian plates. The area of interaction between these two plates is located in the southern Gangdise Mountain and Indian Brahmaputra River valley regions. The Brahmaputra River valley is below an altitude of 4000 m and is a shrub steppe. The higher region consists of subalpine meadows. This diverse geographical environment together with its rich and varied vegetation makes this area the favorite habitat for the Tibetan red deer. Though it is a suitable environment for their survival, migration of individual deer between the different populations exists.

How clear the boundary is between populations depends on the gene flow levels between them. The strength of the gene flow influences a population's genetic diversity, effective size, selection and drift. In addition, it also causes re-distribution of genetic structure. For a heterogeneous population with little or no gene flow, the smaller the local subpopulations are, the faster it loses its genetic diversity and the greater the genetic differences between the local subpopulations. When Nm < 1 genetic drift may result in differentiation them. Microsatellite polymorphism of the Tibetan red deer suggests that the populations in the Zengqi, Woka and Baidui areas all exhibit Nm > 1, indicating gene flows between them. The gene flow between the Zengqi and Woka populations is relatively weak (Nm = 1.67). This may be due to increased human activity in these regions over the past several decades. Specifically, road construction has affected the migration of deer between these two areas. However, the population isolation time is not long from a genetic perspective, so there is no obvious genetic difference ($\Phi st = 0.421$, $p > 0.05$). In addition, the constructed phylogenetic trees show that genetic diversity is relatively high in Tibetan red deer populations. The three geographical units are interlaced together, instead of forming three separate groups, suggesting that there is no obvious differentiation across these populations, which have

a similar genetic basis and significant gene flow between them. We therefore propose to protect and control the Tibetan red deer in these three regions as a single combined management unit.

Conclusion

The Tibetan red deer is an indigenous Chinese species, once on the brink of extinction. These results show that these Tibetan red deer populations have relatively high genetic diversity. We propose to protect and control the Tibetan red deer in these three regions as a single management unit.

Acknowledgments

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