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Authors: Yuan, Yaohua, Huang, Kangning, and Liu, Qunxiu

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Population status and genetic analysis of captive red goral (*Naemorhedus baileyi*) in Shanghai Zoo, China

Yaohua YUAN, Kangning HUANG and Qunxiu LIU*

Shanghai Endangered Species Conservation and Research Centre, Shanghai Zoo, 200335 Shanghai, China; e-mail: liuqunxiu2019@163.com, 346338903@qq.com, knhuang@163.com

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Abstract. The red goral is an endangered species with a small population and narrow distribution in China. The only captive population (21 individuals) is held in Shanghai Zoo, China. Demographic and genetic analyses were carried out to assess the status of this captive population and put forward conservation and management proposals. Population status was assessed on the basis of studbook records from 1981 to 2016. The population size grew from seven (three males, four females) to 31 (19 males, 12 females) in 2012 ($\lambda = 1.06$) and 21 (17 males, four females) in 2016 ($\lambda = 0.91$). From 1981 to 2016, a total of 81 births (45 males, 36 females) and 68 deaths (32 males, 32 females, four unknown) occurred. The mean kinship (MK) of the population is 0.2614 and the average inbreeding index is 0.2037. Parturition mainly occurs in May, June and July, and deaths occurred in January, February, July and November. In addition, the genetic diversity of this population was analysed on the basis of nine microsatellite loci. The results revealed that 14 primer-amplified bands were found; nine of them were stable, with four loci highly polymorphic (polymorphic information content, PIC > 0.05), three moderate ($0.25 < PIC < 0.5$), one low (PIC < 0.25) and one monomorphic (PIC = 0). In total, 34 alleles were detected from eight microsatellite loci, with the number of alleles per locus (A) ranging from 3-6 ($\bar{x} = 4.250$), and the effective number of alleles (Ne) from 1.245 to 3.862 ($\bar{x} = 2.529$). The observed heterozygosity (H0) varied from 0.143 to 0.857 ($\bar{x} = 0.562$), and the expected heterozygosity (He) from 0.201 to 0.755 ($\bar{x} = 0.550$). The polymorphism information content (PIC) varied from 0.188 to 0.705 ($\bar{x} = 0.500$). The results indicated a moderate level of genetic diversity among the red goral population in Shanghai Zoo.

Key words: genetic diversity, population management

Introduction

The red goral (*Naemorhedus baileyi*) belongs to Bovidae, Caprinae, *Naemorhedus*, and is treated as a Grade I national protected species in China, evaluated as Vulnerable (VU) by the IUCN and listed in Appendix I by CITES (CITES 2005, Duckworth & MacKinnon 2008). The red goral is distributed across southeastern Tibet and parts of Yunnan Province, China, and has also been seen in northern Burma, Myanmar and the adjacent area of Assam, India (Shackleton 1997, Wang 1998). In recent years, the wild population of red goral has been drastically reduced as a result of intensive habitat destruction and illegal hunting. According to an investigation in 1988, the wild population of red goral was less than 1500 (Zhang & Zhou 1988, Yin & Liu 1993). The only captive population is held in Shanghai Zoo, China, and this may serve as a source for reintroduction in the future. Only five initial founders were brought from the wild in 1981 (seven individuals were captured, but two of them did not have offspring) and as yet no additional specimens have been introduced. A scientific breeding program based on genetic analysis is, therefore, necessary for the sustainable development of this population. Research on the red goral is based mainly on the captive population; the subjects cover reproductive ecology (Wu et al. 1993, 1996, Wu & Zhang 1993, Guo et al. 2004, Xie 2006), behavioural rhythms and activity (Zheng 2011, Wang et al. 2014), foraging and nutrition (Wang 2014), disease control and treatment (Zhou et al. 2014), welfare (Hou 2014) and other biological information (Liu & Zhang 1994).

In recent years, the Chinese Association of Zoological Gardens (CAZG) has paid increasing attention to captive population management, and a number of taxonomy advisory groups (TAGs) have been organized. Some species have been managed as a priority, including the South China tiger (*Panthera tigris amoyensis*) (Wang et al. 2003), golden snub-nosed monkey (*Rhinopithecus roxellana*) (Yu 2004,

* *Corresponding Author*

Zhao et al. 2016), plains zebra (*Equus quagga*) (You et al. 2017) and Oriental white stork (*Ciconia boyciana*) (Ma 2011). However, the management of the red goral population has received less attention. Based on the studbook of the red goral population recorded from 1981 to 2016, we carried out a demographic analysis of this population.

Conservation genetics is helpful in providing useful information on genetic structure and population status, which are essential for the management of populations in captivity (Zhou et al. 2007). Among the genetic tools, analysis of microsatellite DNA loci is valuable in the study of population genetics and in detecting plant and animal genetic structure, determining parentage and relationships, etc. (Woodhead et al. 2008, Wan et al. 2004). Microsatellite technology has become a powerful tool to investigate genetic diversity and structure, and has been widely used for many species (Zhang et al. 2003, 2005, Wang et al. 2007, Wu et al. 2009, Alasaad et al. 2011, Yamamoto et al. 2013), including many captive species (Arif et al. 2010, Luikart et al. 2011, Yang & Jiang 2011). Based on microsatellite technology, we tested primers originally designed for species closely related to the red goral, *Nemorhedus caudatus*, *Rupicapra rupicapra* and *Rupicapra pyrenaica ornate* (Mead 1989, An et al. 2005, Lorenzini 2005, Cassar et al. 2007) to find microsatellite polymorphic loci in the red goral. We screened stable amplification and variable microsatellite DNA loci of the red goral and assessed the level of genetic diversity of the population in Shanghai Zoo. Based on the demographic and genetic analysis, we discuss the population status of the red goral, and offer suggestions on its population management.

Material and Methods

Research species

Shanghai Zoo has held the only population of red goral since 1981. All gorals are managed in accordance with the technical protocols of husbandry for wild animals issued by the State Forestry Administration, China (LY/T 2981). In total, three areas were designed for daily husbandry, an inner room, holding area and outer area, with laneways to access the other areas. The inner room is 4 × 3.6 × 3 m with a straw-shelf and water trough. The holding area is 3.6 × 6.2 m in size, surrounded by a 2.6 m fence with inward inclined (45° angle) mesh (50 cm width). The outer area is 30 × 28 m, surrounded by a fence 2 m high with inward inclined (45° angle) mesh (50 cm width). Complex wooden shelves were set in the outer area as enrichments. The daily diet of the red goral includes locally sourced grass (69.4 %, replaced by hay in winter), leaves (13.9 %, varying by season), carrots (8.3 %), pellets (5.6 %) and soya bean meal (2.8 %). Salt licks are always present. The pens are cleaned every day and sterilized at three-day intervals. Most fawns are separated from their mothers at approximately three months old. Behaviour enrichment and positive reinforcement training are carried out every day to promote daily husbandry and medical care. Each newborn is marked by subcutaneous implantation of a microchip at less than three months old for individual identification.

Data collection

Data on the population of red goral were collected from 1981 to 2016, and the information about births, deaths and reproduction was summarized. All information was managed using the population management software SPARKS ver. 1.66 (Single Population Analysis & Records Keeping System) (ISIS 2004).

Sample collection and DNA isolation

In 2014, 28 blood samples (2 ml) were collected from each red goral based on chemical anaesthesia (Ketamine, dosage: 3-5 mg/kg and Celazine, dosage: 2 mg/kg) or behavioural training (positive reinforcement stimulation). The samples were stored in EDTA-K2 anticoagulant tubes and shaken manually before refrigeration. For genetic analysis, primers originally designed for *Nemorhedus caudatus*, *Rupicapra rupicapra* and *Rupicapra pyrenaica ornata*, which are closely related to the red goral, were used to find microsatellite polymorphic loci in the red goral (An et al. 2005, Lorenzini 2005, Cassar et al. 2007). The blood samples were analyzed by higher polymorphic loci, and 15 pairs microsatellite primers (BM1818, SR-CRSP08, SY84, SY50, SY242, CSSM66, SY17, SY84B, KCNA44, BM203, SY12A, SY3A, BL42, BMC1009, ILST030Q) were used in higher polymorphic loci analysis. Total genomic DNA was isolated using a Tiangen Kit, extracted with 100 ml TE (pH = 8.0) and preserved at a temperature of –20 °C.

Polymerase chain reaction and microsatellite genotyping

PCR reactions were set up in 10 μL of reaction volume containing 1.0 μL of 10 × PCR Buffer, 0.8 μL of 200 μmol/L dNTPs, 0.20 μL of 10 μM of each primer pair, 0.05 μL of ExTaq DNA Polymerase

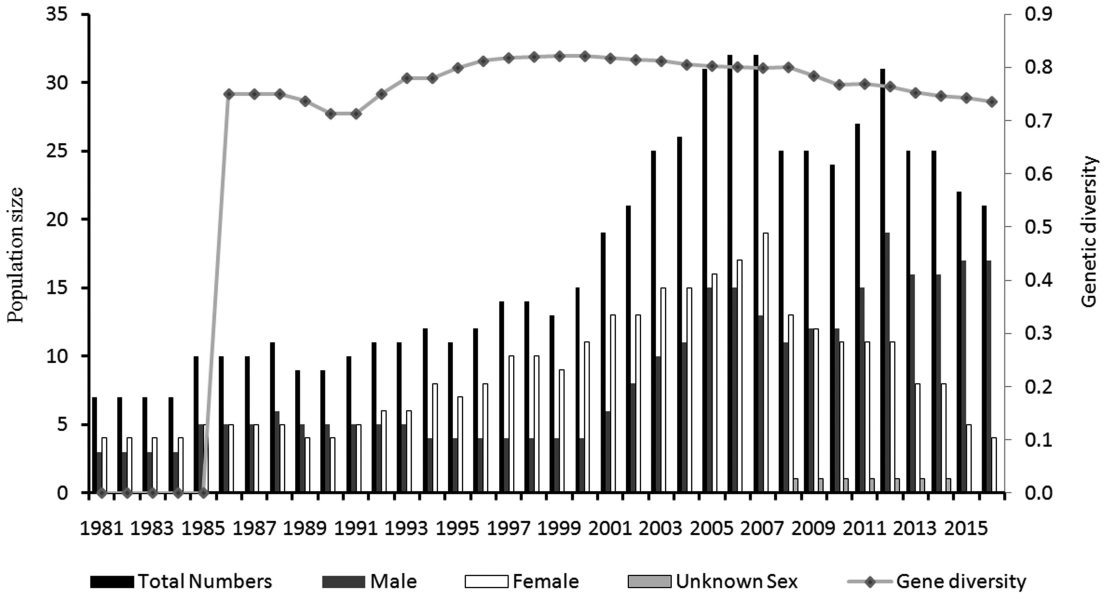

Fig. 1. Dynamics of population and genetic diversity of the captive red goral population from 1981 to 2016.

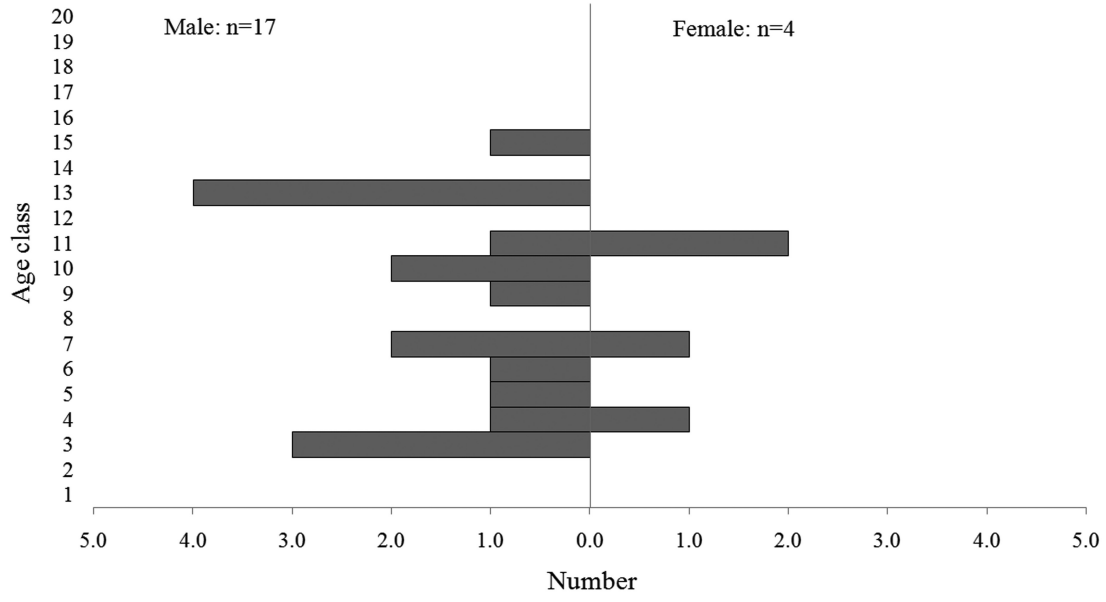

Fig. 2. Age and sex structure of the the captive red goral population in 2016.

(Takara Bio-Engineering Co., Ltd., Beijing, China), 2 μLof DNA elutant (approximately 30 ng) and RNase-free water to make the final reaction volume up to 10 μL. The forward primers were fluorescence labeled with FAM, HEX or NED. PCR amplification was performed on an ABI 9600 Fast Thermo cycler (Applied Biosystems, U.S.A.). The PCR reaction conditions included an initial denaturing step of 95 °C for 5 min, followed by 30 cycles of 98 °C for 10 s, 52 °C to 61 °C depending on the locus for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Amplicons were checked on 1 % agarose gels; after detection of the target band using a genetic analyzer model ABI-PRISM3730 to assess allele size, genotyping by capillary electrophoresis was performed by Shanghai SANGON Biological Technology Co., Ltd., Shanghai, China.

Data analysis

A total of 13 genetic parameters, as well as population diversity (genetic diversity retained through the generations) were calculated using PMx (Lacy et al. 2012). Based on microsatellite analysis, heterozygosity (H0), expected heterozygosity (He), number of alleles, effective number of allelesand polymorphic information content (PIC) were calculated using

Table 1. The causes of death of red goral in Shanghai Zoo from 2009 to 2016.

Death year	Stillbirth	Diarrhoea	Congenital dysplasia	Fighting	Dystocia	Others	Total
2009		4		1		1	6
2010	1	3	1				5
2011		1			1	1	3
2012		1		2		3	6
2013		5					5
2014	2			1			3
2015				1			1
2016						1	1
Total	3 (10 %)	14 (46.7 %)	1 (3.3 %)	5 (16.7 %)	1 (3.3 %)	6 (20.0 %)	30

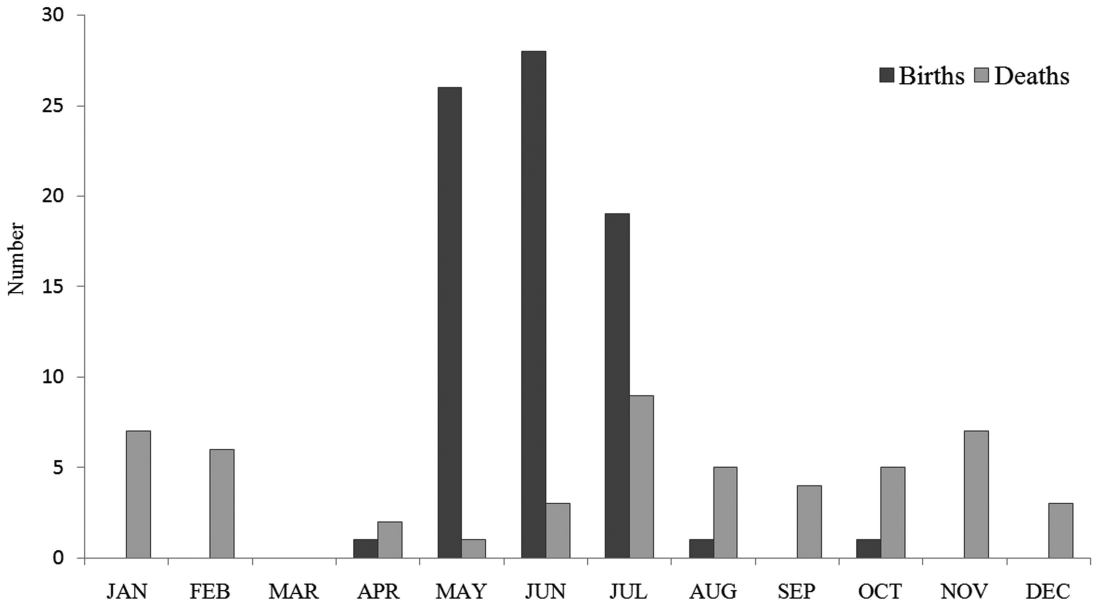

Fig. 3. Births and deaths of the captive red goral population in different months (1981-2016).

CERVUS 3.0 (Kalinowski et al. 2007); F-Statistics (Fis), linkage equilibrium and Hardy-Weinberg equilibrium were calculated using POPGENE 4.0.

Results

Population size

The initial sex ratio of five founders was four (female: male). From 1981 to 2016, the red goral population increased gradually ($\lambda = 1.039$) with a turning point in 2012. Population size varied from seven (three males, four females) in 1981 to 31 (19 males, 12 females) in 2012 ($\lambda = 1.06$), and 21 (17 males, four females) in 2016 ($\lambda = 0.91$) (Fig. 1). A total of 81 births (45 males, 36 females) and 68 deaths (32 males, 32 females, four stillbirths with uncertain sex) occurred. Deaths were mainly caused by diarrhoea (mainly new-borns under three months old), fighting and stillbirth (Table 1). The mortality rate was 25 % for 1-year-old individuals, and 18 % for sub-adults (males: 0-2 years old, females: 0-3 years old).There was no centralized distribution for the age structure of the population (Fig. 2), but the sex ratio is unbalanced (4.25:1).

Reproductive biology

During oestrus, the females reduce food intake, show presenting behaviour and accept male mating. According to the behaviour record, the oestrous period of the red goral is from November to mid-January of the following year, and one fawn is produced per birth. The oestrus duration is 1-2 days, and the gestation period is 220-226 days. During the breeding season, 96.1 % fawns were born in May (34.2 %), June (36.8 %) and July (25 %) (Fig. 3). Deaths mainly occurred in January (13.5 %), February (11.5 %), July (17.3 %) and November (13.5 %). The average lifespan of captive red goral is 10.7 years (male:

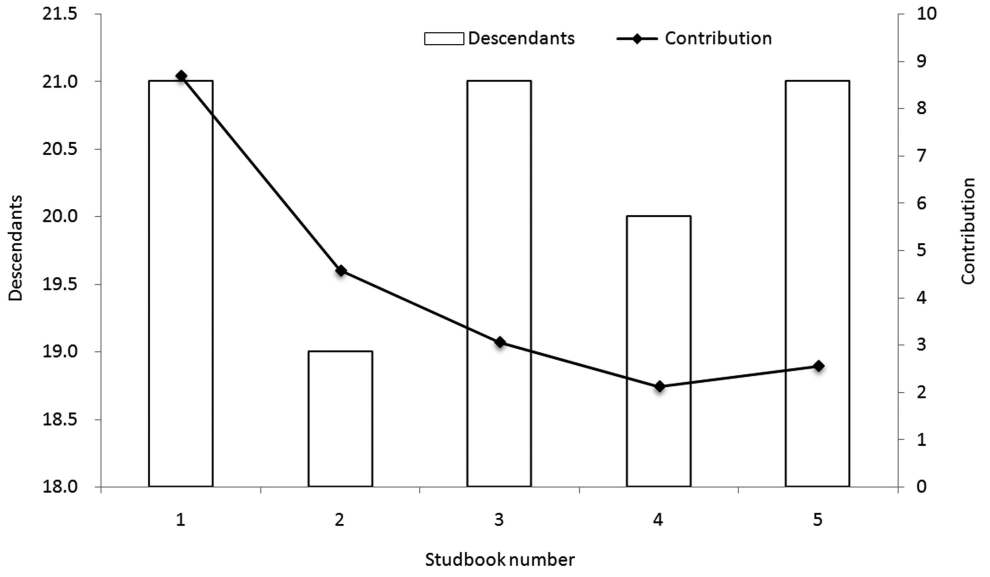

Fig. 4. Contribution of founders and descendants in the captive red goral population.

Table 2. Genetic parameters of the captive population of red goral in Shanghai Zoo.

Parameters	Values
Founders	5
Potential founders	0
Surviving descendants	21
Gene diversity remaining	0.739
Gene value	0.722
Founder genome equivalents	1.91
Founder genomes surviving	3.23
Mean kinship (MK)	0.261
Mean inbreeding	0.204
Generation time (years)	7.50
Mean Ne	9.26
Current Ne	6.00
Ne/N	0.286

max = 22, $\bar{X}$ = 11.2, female: max = 18, $\bar{X}$ = 10.2). Reproductive activity occurs in males from 3 to 15 years old and in females from 4 to 11 years old.

Genetic analysis

There were a total of seven founders in 1981; one died early on and one did not breed. Therefore, five founders, without any additions from the wild, comprised this population (Fig. 4). Up to 2016, the captive population held 73.86 % of the genetic diversity of the initial founders. The genetic contribution of the founders was unbalanced, with 41.37 % of genes from one individual and 58.63 % from the other four individuals. The mean kinship (MK) of the population is 0.26 and the average inbreeding index is 0.2 (Table 2).

PCR products were obtained by amplifying nine microsatellite loci (SR-CRSP08, SY84, SY242, CSSM66, KCNA44, SY12A, BM203, ILST030Q, BMC1009). Three randomly selected PCR products were sequenced to confirm the presence of microsatellite sequence repeats (Table 3). Among the products, one microsatellite locus (BMC1009) was monomorphic, with one allele, and eight loci (SR-CRSP08, SY84, SY242, CSSM66, KCNA44, SY12A, BM203, ILST030Q) were polymorphic, with ≥ 3 alleles per locus (Table 3).

A total of 34 alleles were distributed among the eight loci of the 28 red goral samples examined. The observed number of alleles (A) per locus ranged from 3-6 ($\bar{X}$ = 4.25). The effective number of alleles (Ne) was lower than A and ranged from 1.25 (BM203) to 3.86 (SY12A) ($\bar{X}$= 2.53). The observed heterozygosity (H0) varied from 0.14 (BM203) to 0.86 (SY12A) with a mean of 0.56, and the expected heterozygosity (He) from 0.20 (BM203) to 0.76 (SY12A) with a mean of 0.55. The PIC values of the eight microsatellite loci ranged from 0.19 (BM203) to 0.70 (SY12A), with a mean of 0.50 (Table 2). The results indicate a moderate level of genetic diversity of the red goral population in Shanghai Zoo.

A total of 14 primer-amplified bands were found: nine of them were stable, with four loci highly polymorphic (PIC > 0.05), three moderate (0.25 < PIC < 0.5), one low (PIC < 0.25) and one monomorphic (PIC = 0). The Fis values ranged from –0.2059 to 0.2751 with a mean of –0.0451. Among the eight loci, two

Table 3. The eight microsatellite loci used in the study of red goral.

Loci	Primer (5′-3′)	Repeat unit	Dye	Anneal temp. (°C)	Fragment size (bp)
SR-CRSP08	F:GAACTGAACTTGTTAGTATGTTGGG R:TTGTTATGCTTGATGTTATTTTGTTAC	GT	FAM	53	220~227
SY84	F:GAACTGAACTTGTTAGTATGTTGGG R:TTGTTATGCTTGATGTTATTTTGTTAC	CA	JOE	54	170~188
SY242	F:GTGAGAAATAATACCTCCCTGAAG R:AACATCCAGACCAAAACTTGC	GT	FAM	55	175~194
CSSM66	F:ACACAAATCCTTTCTGCCAGCTGA R:AATTTAATGCACTGAGGAGCTTGG	GT	FAM	56	152~183
KCNA44	F:CTGGAAGAGATGTTAAAAGTA R:CACTGAATAAACAACTGCTCA	TG	JOE	59	218~227
SY12A	F:TTTCTGCTTCGCTGGACC R:AACCCACTTCAGTATTCTTGCTTA	GT	HEX	60	198~216
BM203	F:GGGTGTGACATTTTGTTCCC R:CTGCTCGCCACTAGTCCTTC	TG	FAM	60	214~222
ILST030Q	F:CTGCAGTTCTGCATATGTGG R:GTTTCTTCTTAGACAACAGGGGTTTGG	TG	HEX	61	162~167

Table 4. Genetic information on eight microsatellite loci for the red goral.

Locus	A	Ne	H0	He	PIC	Fis	HW (*P*-value)	FNULL
SR-CRSP08	3	1.682	0.464	0.413	0.369	–0.1447	0.8868	–0.0670
SY84	5	3.853	0.857	0.754	0.701	–0.2059	0.9278	–0.0759
SY242	4	1.762	0.429	0.440	0.398	–0.0079	0.4996	0.0017
CSSM66	6	3.280	0.821	0.708	0.647	–0.1709	0.9364	–0.0880
KCNA44	5	2.554	0.464	0.619	0.553	0.2369	0.0243	0.1309
SY12A	5	3.862	0.857	0.755	0.705	–0.1253	0.7040	–0.0908
BM203	3	1.245	0.143	0.201	0.188	0.2751	0.1060	0.1506
ILST030Q	3	1.992	0.464	0.507	0.440	0.0679	0.0122	0.0042
Average	4.250	2.529	0.562	0.550	0.500	–0.0451		

Table 5. Analysis of linkage disequilibrium of eight microsatellite loci for the red goral.

Locus	SR-CRSP08	SY84	SY242	CSSM66	KCNA44	SY12A	BM203	ILST030Q
SR-CRSP08		0.228900	0.000354	0.025464	0.738128	0.428864	0.800340	0.793816
SY84			0.418186	0.055188	0.935920	0.007050	0.849334	0.508766
SY242				0.603800	0.354712	0.037220	0.209352	0.174248
CSSM66					0.719052	0.047976	0.959992	0.577244
KCNA44						0.119432	0.285670	0.016658
SY12A							0.024304	0.033064
BM203								0.735396
ILST030Q								

(KCNA44, ILST030Q) significantly deviated from Hardy-Weinberg equilibrium ($P < 0.01$) (Table 4). The population showed seven instances of combined linkage disequilibrium ($P < 0.05$), and one (SY242, SR-CRSP08) remained after Bonferroni correction ($P = 0.006 < 0.05$) (Table 5).

Discussion

The small red goral population in Shanghai Zoo is influenced by its birth rate and mortality rate, with unexpected events also contributing to population extinction (Ma & Li 2003). Small populations generally have low genetic diversity due to inbreeding,

no complementarities and genetic drift. When population density is below a certain threshold value, Allee effects may also be more likely. Therefore, population management based on genetic analysis is urgently required. The mortality rate of sub-adult gorals is much higher than that of other age groups. In the last decade, the percentage of still births was 10 %, 50 %, and 16.7 % of deaths were caused by diarrhoea and fighting, respectively. For this red goral population, the contribution of the five founders to the descendants was unbalanced, which led to a lack of genetic diversity and extreme inbreeding. Inbreeding may serve as the main reason for the high rates of stillbirth and mortality, resulting in a decline in the population. Comparison of the past and current goral population in Shanghai Zoo showed that an increase in males intensified competition, resulting in the high mortality rate. In the limited space available in Shanghai Zoo, only a small percentage of males can be involved in breeding and other individuals are separated (Kleiman et al. 2010); therefore, males with potential genetic advantages may not reproduce and contribute to the population.

Shanghai Zoo holds the only captive red goral population that has been established for more than 30 years with no additions from the wild. According to Ardrenet al. (1999), small populations may be subject to inbreeding, null alleles, and low heterozygosity (Lade et al. 1996, Wu et al. 2008). However, for the red goral population in this study the Fis value is –0.0451, not significantly different from zero, which indicates that the population may not have suffered severe adverse effects of inbreeding. Furthermore, a negative Fis value means that null alleles may not exist (Wu et al. 2008). In this study, two microsatellite loci significantly deviated from Hardy-Weinberg equilibrium ($P < 0.01$) and a pair of loci deviated from linkage disequilibrium. For this small captive population with few founders, inbreeding may have led to loci being in Hardy-Weinberg disequilibrium and linkage disequilibrium. In our study, the frequency of null alleles was less than 0.2, so the data could be used for population genetic analysis (Wen et al. 2013). The population was derived from an original five founders, and it is almost impossible to obtain more individuals from the wild. Inbreeding and lack of gene flow may serve as severe threats to this population. Therefore, it is important to implement scientific, especially genetic, management to promote the healthy development of the group.

According to Botstein et al. (1980), the genetic polymorphism of a microsatellite locus is high if PIC > 0.5, medium if 0.5 < PIC < 0.25 and low if PIC < 0.5. In our study, SY84, CSSM6, KCNA44 and SY12A were highly polymorphic loci, SR-CRSP08, SY242 and ILST030Q were of intermediate polymorphism, BM203 showed low polymorphism and the PIC value of BMC1009 was zero. Therefore, these eight microsatellite loci can serve as efficient genetic markers for the genetic analysis of the red goral population. Genetic diversity is one of the key issues in the study of biological diversity and evolutionary biology (Hartl & Clark 2000). Heterozygosity is an important indicator in the assessment of genetic polymorphism (Nei et al. 1975), and may be influenced by sample size. The average expected heterozygosity of the red goral was 0.550. Compared with other species, long-tailed goral: 0.577 (An et al. 2005); Tibetan antelope: 0.673 (Chen 2005); Arabian antelope: 0.565 (Arif et al. 2010); Sambar deer: 0.44 (Say et al. 2003), the microsatellite polymorphism of the red goral population was evaluated as intermediate.

One successful case of captive population recovery in China, similar to the red goral population, is the South China tiger (*Panthera tigris amoyensis*). All captive South China tigers are derived from six original founders from the wild, and began to breed in the mid 1980s. In 1995, an international symposium on the *ex situ* conservation of Chinese tigers was held in Suzhou, China. Based on the husbandry conditions and reproduction of captive South China tigers, several key decisions were made at this conference: 1) establish a conservation and coordinating committee for the South China tiger; 2) formulation of a five-year (1996-2000) plan for the captive South China tiger population; 3) set a population growth target at 150. From then on, a conference for South China tiger conservation was organized to discuss mating and assignment plans for the following year (the "Convention on *ex situ* conservation of the South China tiger" was formulated to ensure the execution of all decisions assented to by each Zoo). From 2004, a blood sample was collected from each newborn tiger to assess breed purity, and kinship and genetic analysis. At the end of November 2018, the captive population of South China Tiger was 178 (88♂, 89♀, 1 uncertain).

Based on the condition of the captive red goral population and positive experience with the South China tiger population recovery, we propose suggestions for the sustainable development of the red goral population. 1) Scientific research is crucial for the conservation and recovery of the captive red goral population. Better understanding

of the biology and reproductive characteristics of the red goral is necessary for routine husbandry, breeding and welfare. In addition, genetic analysis, as a scientific approach for population conservation, should be carried out on each newborn goral, so that the studbook can be accurately updated every year. 2) Based on the genetic analysis and studbook records, a mating and assignment plan should be established annually, as well as a long-term population growth and genetic diversity target. 3) All resolutions should be strictly executed once a consensus is reached. 4) To promote the breeding of red goral, artificially assisted reproduction may serve as a critical measure to promote population growth. In Shanghai Zoo, there has been a technological breakthrough in semen collection by electro-ejaculation and semen freezing for the red goral. More studies are being carried out on artificial insemination.

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