RESEARCH ARTICLE

Microsatellite variation in China's Hainan Eld's deer (*Cervus eldi hainanus*) and implications for their conservation

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Abstract Hainan Eld's deer (*Cervus eldi hainanus*) experienced a dramatic decline in the late 1960s through early 1970s and by 1976 only 26 deer remained in Datian of Hainan Island, China. Since then, conservation efforts have successfully rescued this deer from extinction. We employed 10 microsatellite DNA loci to index genetic variation in the one source (Datian) and two introduced populations (Bangxi and Ganshiling) and suggest implications for the conservation of the species. A total of 40 alleles at 10 loci were examined from 198 deer blood samples. The source population harbored all 40 alleles, while the Bangxi and Ganshiling translocated populations contained 24 and 26 alleles, respectively. The genetic variability was low ($H_e \approx 0.33$) for each of the three populations. No significant difference in genetic variability between the three populations was detected ($P > 0.05$); yet significant differentiation was found among the three populations. Our results suggest that founder effects and genetic drift have affected the two translocated populations. For conservation we recommend the three populations be managed as a meta-population. When establishing future reintroductions, the founder population should have a size larger than the original 26 founders in Datian population or be composed of a cohort of over 20 same-age individuals with 1:1 sex ratio. Genetic monitoring for both the source and translocated populations should be continuously conducted in order to assess the effectiveness of deer conservation in the future.

Introduction

Eld's deer, or brow-antlered deer (*Cervus eldi*), is a medium-sized tropical deer. Its four subspecies historically occurred throughout much of southeast Asia, extending from Manipur in eastern India to Indochina and southern China (Fig. 1). Habitat destruction and fragmentation, as well as overexploitation have greatly reduced the population size of all subspecies (McShea et al. 1999; Bhumpakphan et al. 2003; Dang and Thuy 2003; Johnson et al. 2003; Singsit 2003; Weiler 2003; Zeng et al. 2005). At present, the species is listed in Appendix I of the Convention on International Trade in Endangered Species (CITES) and is considered endangered by the World Conservation Union (IUCN).

Hainan Eld's deer (*C. e. hainanus*) only occurs in Hainan Island, China (Zeng et al. 2005). Historically, this deer was distributed over the whole Island, except the mountainous region in the center of the Island (Zeng et al. 2005). Farmland encroachment, caused by continuous immigration of people from the mainland, and over-hunting had driven the deer population to decline to ~500 individuals by the early 1950s (Zeng et al. 2005).

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Fig. 1 Distribution range of *Cervus eldi* in southeast Asia and its sampling locations (BNR, DNR and GNR) in Hainan Island. The distribution information of the species is from McShea et al. (1999), Bhumpakphan et al. (2003), Dang and Thuy (2003), Johnson et al. (2003), Singsit (2003), Weiler (2003), Zeng et al. (2005). *BNR*: Bangxi Nature Reserve; *DNR*: Datian Nature Reserve; *GNR*: Ganshiling Nature Reserve
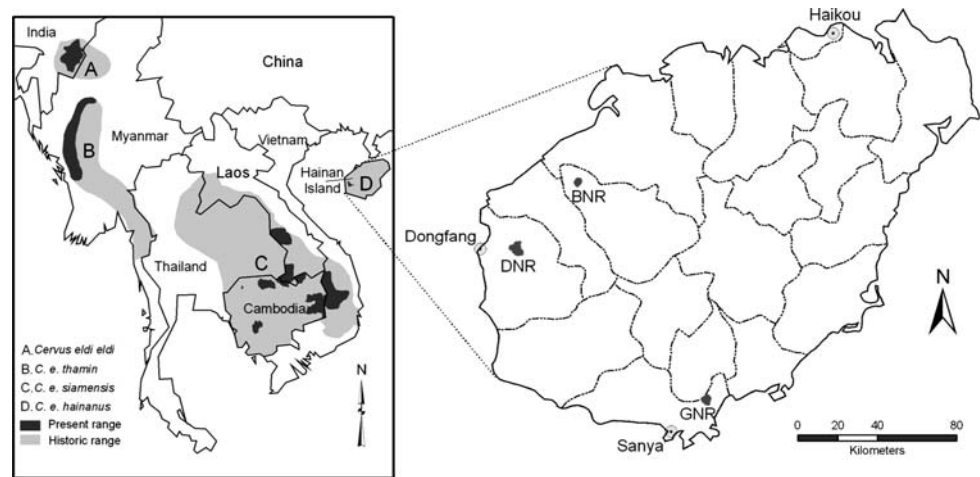
Population decreases continued until 1976, when only 26 deer remained in the far west region of the Island, in an area named Datian (25–30 km^2).

A population restoration programme was launched in 1976 with the establishment of Datian Nature Reserve (DNR). The deer population increased to 151 by 1986, however, a portion of the population (86 deer) were within a small enclosure of 1 km^2 to avoid extinction of the whole population from poaching (Song and Li 1995). The enclosure was enlarged to 3 km^2 in 1988 and to 13.14 km^2 (the current total size of DNR) in 1995. This last expansion resulted in reuniting the deer within and outside of the enclosure. Deer were free ranging within the enclosure and there was no breeding management. In 2000, the Datian population size was estimated at 864 individuals (Yuan et al. 2001). Due to a high population density within the fenced DNR, further translocations of deer to available habitat are under consideration by the local authorities.

Two translocated populations were founded in Bangxi in 1990 and in Ganshiling in 1997, respectively. In total, 18 fawns were introduced to Bangxi during 1990–1992 and 20 fawns were introduced to Ganshiling in 1997 (Fig. 1; Zeng et al. 2005). Until now, no genetic information has been collected from the translocated deer, nor have genetic management actions been undertaken for these groups. In 2002, Bangxi and Ganshiling held 115 and 41 deer, respectively (Zeng et al. 2005).

In 2003 it was suggested that Hainan Eld's deer be used to supplement populations of the endangered subspecies *C. e. siamensis* in Thailand (Pukazhenthi 2003). If Hanain Eld's deer are to be considered for this purpose, their reproductive and genetic status should be assessed prior to supplementation. In Hainan Eld's deer, there is no evidence of decreased fecundity, and no observed anomalous neonatal and juvenile mortality have been recorded (Song and Li 1990). One study on genetic diversity indicated that all 55 Hainan Eld's deer sampled from the DNR shared an identical mtDNA haplotype (Pang et al. 2003). Meanwhile, Pang et al. (2003) used a simulation approach to test the likelihood of various bottleneck scenarios; their results showed that the deer had probably gone through a mild bottleneck in the early 1960s followed by a severe bottleneck in the late 1960s or early 1970s.

Empirical studies suggest that increased inbreeding can lower individual fitness and the probability of population persistence (O'Brien et al. 1987; Ralls et al. 1988; Ballou 1997; Saccheri et al. 1998). In captivity, inbred *C. e. thamin* suffered greatly increased juvenile mortality (Ralls et al. 1979), and *C. e. siamensis* possessed an extremely high neonatal mortality (90%) (Mauget et al. 2001). The genetic study of the Eld's deer logically led to serious consideration of whether a small, inbred and isolated population can remain viable without the introduction of new genetic material from other populations (Balakrishnan et al. 2003). However, it is dangerous to make management decisions based only on mtDNA, because mtDNA has certain limitations as a genetic marker for estimating population genetic diversity (Zhang and Hewitt 2003). For example, mtDNA represents only a single locus and reflects only the history of female lineages; moreover, the effective population size of mtDNA is only a quarter of that of nuclear autosomal sequences. Thus, nuclear DNA polymorphisms are necessary to reveal the genetic diversity and population genetic structure from both sexes within populations.

Here, we report the results of a study employing 10 microsatellite DNA markers to index genetic variation in one source and two introduced populations of Hainan Eld's deer. Our objectives were to (1) ascertain the genetic diversity of the source population (DNR) as background information for further translocation in China or abroad; (2) evaluate the genetic consequences of the ex situ conservation programme; and (3) provide recommendations for species conservation.

Materials and methods

Sample collection

Blood samples ($n = 198$) were taken from three deer populations in 2003 (Fig. 1). Among them, 159 samples, including 132 adults (92 males, 40 females) and 27 fawns (14 males, 13 females), were obtained from the Datian population (DNR), 19 adults samples (9 males, 10 females) were collected from the Bangxi population (BNR), and 20 adults samples (10 males, 10 females) were obtained from the Ganshiling population (GNR). All the sampled deer were tagged and blood samples were taken and stored in DNA preservation buffer [0.1 M Tris–HCl (pH 8.0): 0.1 M EDTA (pH 8.3): 1% SDS, volume ratio of blood and buffer 1:1] and preserved at –20°C.

DNA extraction and amplification

DNA was isolated from blood samples, using a standard phenol–chloroform method (Sambrook et al. 1989). We surveyed 10 microsatellite loci identified from species of Bovidae and Cervidae and known to be polymorphic in the Hainan Eld's deer (Zhang et al. 2005). Polymerase chain reaction (PCR) amplifications were performed in Perkin Elmer GeneAmp 9700 thermal cyclers using the following conditions: a total reaction volume of 10 μl containing 20–30 ng of genomic DNA, 0.3 μM of each primer, 200 μM of dNTP, 1.5 mM of Mg^{++}, and 0.3 U of Taq polymerase (Promega) was denatured at 94°C for 4 min, then followed by 40 cycles of 20 s at 95°C, 30 s at 43–55°C (depending on primers, see Zhang et al. 2005), and 20 s at 72°C. The reaction was terminated by a final extension of 2 min at 72°C. Primers were end-labeled with a fluorescent dye, either 6-FAM or HEX and examined on an ABI PRISM® 3100 automated sequencer using GENESCAN® 400HD (ROX) as the internal size standard.

Data analysis

Estimates of allele richness (A_R, El Mousadik and Petit 1996), the number of alleles per locus in each population (A), observed heterozygosity (H_O) and expected heterozygosities (H_E) (Nei 1987), and estimators of F statistics (Weir and Cockerham 1984), were calculated using the computer program FSTAT version 2.9.3 (Goudet 2001). The mean number of alleles for each population and allele frequencies for each locus in each population were estimated using MStools 3 (Park 2001, in preparation). The significance of differences in genetic diversity between the three populations was tested using a paired t test on H_E and A_R values at individual loci (Nei 1987; Leberg 1992). Individual pairwise relationships were assessed in KINSHIP v.1.0 (Goodnight

and Queller 1999) by determining the likelihood ratio of a pair of autosomal microsatellite genotypes for a specified primary and null relationship and attributing significance by simulation. Significances of parent–offspring, full-sib and half-sib relationships were tested under the null hypothesis of pairs being unrelated. Type II error was assessed based on the distribution of 1,000 simulated ratios under the tested hypothesis.

The tests of Hardy–Weinberg equilibrium (HWE) for each population and linkage disequilibrium were performed using the programs ARLEQUIN 2.0 (Schneider et al. 2000). A sequential Bonferroni correction for multiple tests was applied to all estimates of statistical significance (Rice 1989). The significance of population differences was tested using the exact test of population differentiation proposed in GENEPOP 3.4 (Raymond and Rousset 1995a) (dememorization 10,000, batches 100, iterations per batch 5,000). All probability tests were performed applying the Markov Chain algorithm (Guo and Thomson 1992; Raymond and Rousset 1995b).

Results

Genetic diversity and differentiation within and among populations

A total of 40 alleles at 10 loci were examined from 198 deer blood samples. The samples from the source (DNR) population contained 40 alleles, while those from the Bangxi and Ganshiling translocated populations contained 24 and 26 alleles, respectively. All alleles at 3 loci (ILS-TS005, URB58, INRABERN185) were found in each of the three populations. One locus (LSCV085) was found to be monomorphic in the two relocated populations, and not in the source population (Fig. 2).

Heterozygosity and genetic polymorphism displayed low levels in the three populations. The expected heterozygosity (H_e) ranged from 0.2898 to 0.3612 (Table 1). Paired t tests found no significant differences between the three tested populations (t value is 0.77, 1.58, and 1.68, $P > 0.05$). The mean number of alleles per locus (A) was 2.40 ± 0.70 in Bangxi, 2.60 ± 0.84 in Ganshiling and 4.00 ± 1.41 in Datian population (Table 1). However, the value of A might be influenced by the large differences in sample size (159 samples at Datian versus 19 and 20 at the other two sites). The Bangxi population showed a lower value of mean allelic richness than both populations at Datian and Ganshiling (A_R, 2.400 vs. 2.684; 2.400 vs. 2.595, respectively), but no significant difference was detected between the three examined populations (t value is 3.21, 1.50, and 2.69, $P > 0.05$). A negative value of F_{IS} within the three populations was detected in our study

Fig. 2 Frequency histograms for 10 microsatellite loci typed in three populations. *B*: Bangxi population; *D*: Datian population; *G*: Ganshiling population
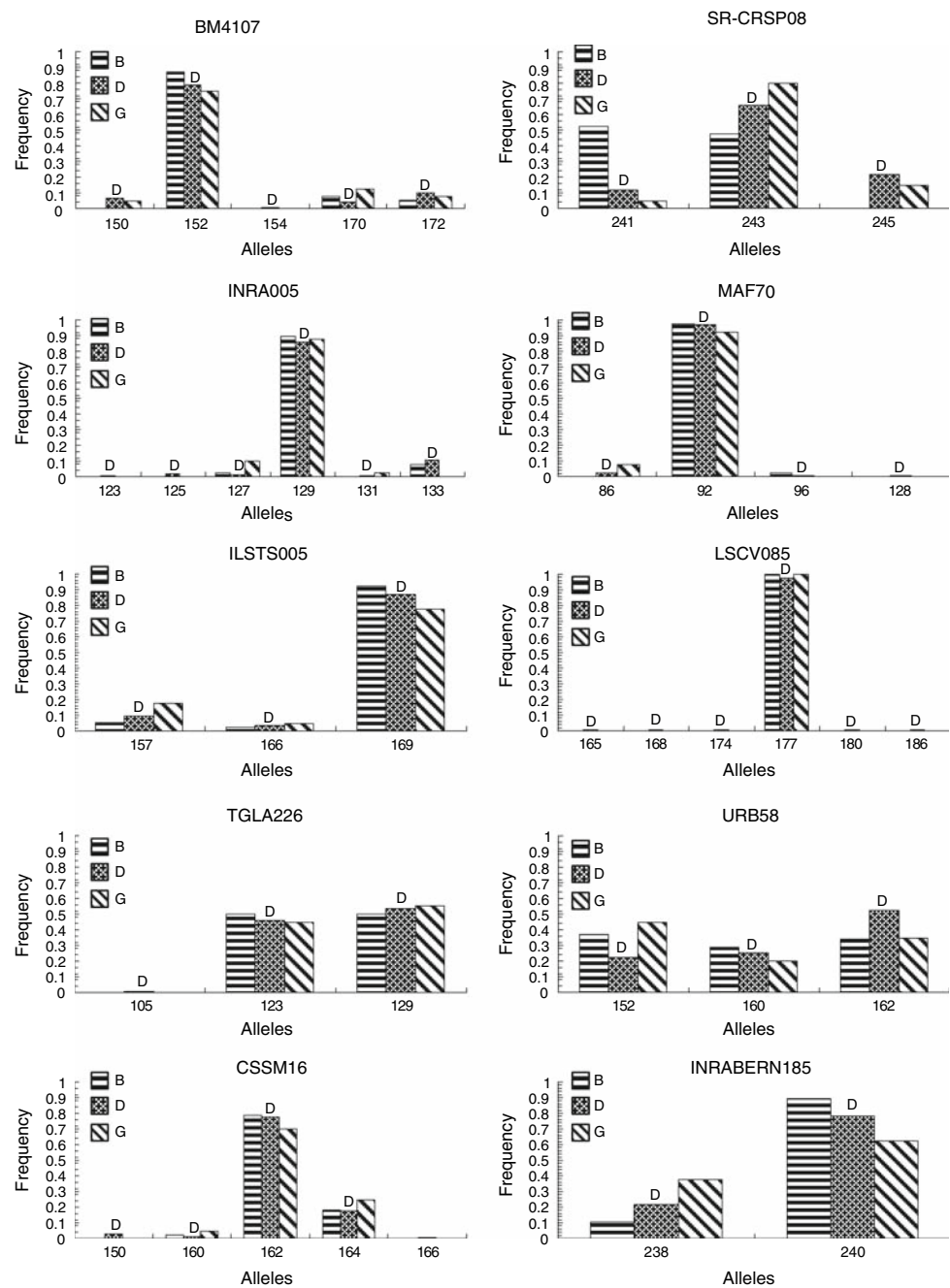

Table 1 Summary of genetic statistics of the three Eld's deer populations on Hainan Island in China

Population	N	A (SD)	A_R (SD)	H_e (SD)	H_o (SD)	n	F_{IS} (SD)	CI
Bangxi	19	2.40 (0.70)	2.400 (0.70)	0.2898 (0.0193)	0.3421 (0.0344)	1	–0.1866 (0.1424)	(–0.2013, –0.1791)
Datian	159	4.00 (1.41)	2.684 (0.68)	0.3280 (0.0077)	0.3346 (0.0118)	0	–0.0203 (0.0432)	(–0.0208, –0.0198)
Ganshiling	20	2.60 (0.84)	2.595 (0.84)	0.3612 (0.0208)	0.3650 (0.0340)	1	–0.0109 (0.1107)	(–0.0217, –0.0001)

Number of samples (N), average number of alleles/locus (A) and standard deviation (SD), mean allelic richness (A_R) per population, expected (H_e) and observed (H_o) heterozygosities, number of monomorphic loci (n), inbreeding coefficient (F_{IS}), 95% confidence intervals (CI) of F_{IS}

(Table 1). KINSHIP analysis revealed 873 significant parent–offspring relationships, 209 significant full-sib relationships and 609 significant half-sib relationships in Datian population, 6 significant parent–offspring relationships, 1 significant full-sib relationships and 7 significant half-sib relationships in Ganshiling population, 3 signifi-

cant parent–offspring relationships, 2 significant full-sib relationships and 3 significant half-sib relationships in Bangxi population ($P < 0.05$, Table 2). The Type II error rate ranges for parent–offspring, full-sib and half-sib were 0.400–0.508, 0.407–0.504, 0.746–0.783, respectively.

The estimate of F_{ST} (Weir and Cockerham 1984) ranged from 0.0202 to 0.0658. After Bonferroni corrections for multiple tests, significant differentiation existed between Bangxi and Datian populations, and no significant differentiation was detected between Ganshiling and Datian populations (Table 3). However, the exact tests indicated that strong differentiation existed between the two translocated populations and the source population (after Bonferroni correction; Table 3). The inconsistency resulted from the power of different statistical approaches. For multi-allelic loci such as microsatellites, the exact test is robust and generally provides high resolving power (Ryman et al. 2006). Thus we believed that significant differentiation existed between the two translocated populations and the source population (after Bonferroni correction).

Hardy–Weinberg equilibrium and linkage disequilibrium

After sequential Bonferroni corrections for multiple tests (Rice 1989), five loci (BM4107, INRA005, ILSTS005, TGLA226, LSCV085) deviated from HWE in the Datian population, whereas only one locus (TGLA226) deviated from HWE in the Ganshiling and Bangxi populations. With Bonferroni corrections, no locus showed linkage disequilibrium in any population.

Discussion

Genetic diversity

Over the last 200 years, Hainan Eld's deer has suffered a dramatic population reduction throughout its range and undergone a long-term demographic bottleneck, which coincided with large numbers of humans immigrating to Hainan Island (Zeng et al. 2005). In addition, two bottlenecks during the 1960s and 1970s have been detected by mtDNA examination (Pang et al. 2003). As predicted by

Table 2 Number of pairwise relationships inferred from multilocus genotypes in three Eld's deer populations

Relationship	Bangxi	Datian	Ganshiling
Parent–offspring relationships	3	873	6
Full-sib relationships	2	209	1
Half-sib relationships	3	609	7

Table 3 Genetic differentiation between populations

	Bangxi	Datian	Ganshiling
Bangxi	–	0.0394*	0.0658*
Datian	**	–	0.0202
Ganshiling	0.00014*	0.00234*	–

Above the diagonal is pairwise estimates of F_{ST} and below is the differentiation significance estimation (exact test) based on allele frequency differences

* After sequential Bonferroni correction for multiple tests (Rice 1989), P value is significant

** High significant (χ^2: infinity), $P < 0.00001$

theories of genetic drift, the reduction in population size appears to have greatly reduced genetic variation (Nei et al. 1975). The level of the Hainan Eld's deer genetic diversity ($H_e \approx 0.33$ for each population, Table 1) is comparable with other deer populations that experienced severe reductions in numbers [e.g. the North American elk (*C. elaphus*), $H_e = 0.34$, Polziehn et al. 2000; Japanese sika deer (*C. nippon*), $H_e = 0.35$, Goodman et al. 2001], and other bottlenecked large mammal populations that have been assessed with microsatellite DNA markers [e.g. cheetahs (*Acinonyx jubilatus*), $H_e = 0.39$, Menotti-Raymond and O'Brien 1995; koala (*Phascolarctos cinereus*), $H_e = 0.33$, Houlden et al. 1996]. Although these studies may not be entirely comparable because different marker systems were employed, it is still suggestive of the level of genetic diversity of the species. Compared with subspecies *thamin* and *siamensis* who still harbor a substantial amount of genetic diversity (Balakrishnan et al. 2003), the genetic data for Hainan Eld's deer are consistent with a substantial loss of genetic diversity (Pang et al. 2003; Balakrishnan et al. 2003), and are similar to data for *C. e. eldi* who is also known to have suffered a severe bottleneck and whose current population was founded by about 20 individuals (Balakrishnan et al. 2003). The deer on Hainan Island are believed to have originated from the southeast Asian mainland and arrived via a land bridge during the end of the Pleistocene and early Holocene (18,000–8,500 years BP, Bhumpakphan et al. 2003). If this is true, the Hainan Island deer population would likely have experienced founder events and genetic drift since that time. This deduction might explain why all 55 females sampled shared an identical mtDNA haplotype (Pang et al. 2003).

The Datian population was divided into two groups in 1986 by an enclosure, and combined in 1995. Based on this history, the observation that five of ten loci deviated from HWE may be caused by a Wahlund effect, however, excess of heterozygotes shows that Wahlund effect is unlikely the reason. We hypothesize that the departure from HWE in all populations is probably attributable to a combination of several possible reasons. One possible explanation is that

the species is polygynous (Zeng, unpublished observation). This mating system is expected to produce heterozygote excess in progeny ($F_{IS} < 0$) relative to HWE because when the effective number of breeding males is small, frequencies of paternally and maternally contributed alleles will differ due to binomial sampling error. In fact, excess of heterozygotes in small populations has been examined in many studies (Robertson 1965; Rasmussen 1979; Falconer 1981; Pudovkin et al. 1996; Luikart and Cornuet 1999). Another explanation for the deviation from HWE is potential biases due to sampling related individuals. Our data showed that a total of 1082 (873 + 209), 7 (6 + 1) and 5 (3 + 2) paired individuals were first order relatives in the Datian, Ganshiling, and Bangxi populations, respectively (Table 2). The third possibility for deviation from HWE is genetic drift in small populations since all the three populations were found by a small number of founders. In addition, sex biases in sampling may have also been shown to influence estimates of genetic diversity (Latch and Rhodes 2006).

Many species with polygynous mating systems may produce individual broods that consist of a mixture of full-sib and half-sib offspring (Dewoody et al. 2000). Small effective population size can result in an increased occurrence of half and full sibs, leading to significant pairwise genetic-relatedness coefficients (Launey et al. 2001). KINSHIP analysis revealed that a total of 818 (209 + 609), 8 (1 + 7) and 5 (2 + 3) paired samples were full-sib and half-sib relationships in the Datian, Ganshiling, and Bangxi populations, respectively (Table 2). Negative F_{IS} values were observed in our study, not surprising when compared to other large mammals with a similar management history. For example, the wild wolf (*Canis lupus*) population on Isle Royale in Minnesota USA was founded by two individuals, but no direct evidence of demographic effects of inbreeding was detected 50 years later (Wayne et al. 1991; Peterson et al. 1998; Liberg et al. 2005). This indicates that F_{IS} is only a technical parameter that examines whether there is random mating (HWE) within the population analyzed. Thus, a detailed analysis of inbreeding, such as a careful pedigree analysis, should be undertaken in the future.

Genetic differentiation

No significant difference in allelic diversity or genetic diversity was detected between the source population and translocated populations. However, slight differences were observed in allele frequencies among these populations; for example, the low frequency allele in the source population was the most common allele in the Bangxi or Ganshiling translocated population (e.g. allele 152 of the locus URB58). A similar situation was observed for the sika deer population in the Japanese archipelago (Goodman et al. 2001). We also found that the two translocated populations were significantly differentiated from the source population. These observations imply nascent signs of founder effects and genetic drift resulting from sampling error when selecting individuals in the relocation process.

At the same time, the Bangxi population is more differentiated from the source population than Ganshiling population (the F_{ST} value between the Bangxi and Datian populations is larger than that between the Ganshiling and Datian populations) and Bangxi population is also significantly differentiated from the Ganshiling population (after Bonferroni correction). Potential explanations for this difference include small founder size and uneven sex ratio of the Bangxi population. This population was initiated from 18 founders who were introduced over a three-year period: 5 fawns (4 males, 1 female) in 1990, 8 fawns (5 males, 3 females) in 1991, 5 fawns (1 male, 4 females) in 1992. Because this species can reproduce at 2 years old (Song and Zeng 2003) and one dominant male can monopolize all the mating (Yuan et al. 1988; Zeng et al. 2001), this relocation procedure caused the effective population sizes to be only one pair during the first breeding year and no more than 10 individuals (2 male and 8 females) in subsequent 2 years (Zeng 2006, Personal communication). In contrast, the founders in the Ganshiling population were 20 fawns with equal sex ratio, resulting in a larger effective population size for this population compared to the Bangxi population.

Conservation implications

Although the population restoration programme for Hainan Eld's deer has been successful at increasing population size, our finding of low genetic diversity is complimented by previous mitochondrial DNA studies (Pang et al. 2003) and suggests that bottleneck and genetic drift processes are affecting this species. Several conservation implications arise from our conclusion.

First, the three populations we surveyed should be managed as a meta-population. Although the two translocated populations are differentiated from the source population, exchanging genetic material across populations may help to ensure the long-term viability of regional populations and the species as a whole. With the relatively large population size, the deer in DNR should provide demographic reservoir for further conservation. Exchanging individuals among the three populations will minimize the loss of genetic variability through genetic drift. As mentioned above, Hainan Eld's deer is polygynous, one male could potentially mate with more than one female in one breeding season. Thus a male will contribute more genetic material to the population in a shorter period than a

female. There are reports that the polygynous mating system can accelerate the increase of co-ancestry among individuals within breeding groups most rapidly in the absence of migration and when high levels of reproductive variance exist among males (Chesser 1991a, b). Consequently, relationships between offspring from individual males would be at least at the level of half-siblings. Therefore, exchanging males between populations should be an effective way to minimize inbreeding. Song (1996) determined that translocating 1% of males between any two Hainan Eld's deer populations could greatly mitigate the loss of genetic heterozygosity based on population viability analysis. To minimize the loss of genetic diversity, we recommend translocating prime males to the Bangxi and Ganshiling populations, since only successfully reproducing immigrants can contribute to the genetic makeup of translocated populations (Vila et al. 2003).

Second, the large genetic differentiation between the Bangxi population and the source population resulted from ignoring the potential affects of founder population structure on the effective population size. In future translocations, the founder population structure should be of particular concern. Based on our results, the founder population should have both a balanced genetic composition and a large effective population size. To establish a viable population, it is recommended that a minimum of 20–30 genetically effective founders be used (Frankham et al. 2002), that the founder population be greater than the original 26 founders in Datian, or that the translocated population be composed of a cohort of over 20 same-age individuals with 1:1 sex ratio, which is based on our data from the Ganshiling population.

Third, Griffith et al. (1989) indicates that reintroducing species throughout their historical or former range is more successful than translocations to areas where the species did not occur before. Therefore, we suggest that Hainan Eld's deer be reintroduced at more sites within their historical distribution throughout Hainan Island.

Finally, we recommend that genetic monitoring for both the source and translocated populations be continuously conducted in order to assess the effectiveness of the conservation programme. Many factors affect the success of reintroductions and management programmes for endangered species. Often these factors interact, and based on our findings it is clear that a species' biology, history, ecology and genetic makeup should be considered holistically. An appreciation of all these factors will result in more effective management strategies for the Hainan Eld's deer in the future.

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