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Full Length Research Paper

Microsatellite variability reveals significant genetic differentiation of giant pandas (*Ailuropoda melanoleuca*) in the Minshan A habitat

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Although, Minshan A habitat is an area with one of the largest numbers of wild giant pandas (*Ailuropoda melanoleuca*); it may be threatened by habitat loss and fragmentation. In this study, 10 microsatellite DNA markers were used to assess population genetic structure of giant pandas from two critical reserves (Tangjiahe and Wanglang) in the Minshan A habitat. The results revealed high levels of genetic differentiation (F_{ST} = 0.134) between the two populations. This differentiation was supported by the assignment tests using the Bayesian clustering method in STRUCTURE. The uniqueness of the populations was also supported by private alleles. This indicated a significant population fragmentation in Minshan A region. In addition, the high individual inbreeding coefficients for Wanglang indicated increased levels of homozygosity in the wild populations. Fortunately, those populations had high levels of genetic diversity. The average allelic richness (*AR*) and expected heterzygosity (H_E) were 4.520 and 0.689, respectively for Tangjiahe and 4.584 and 0.648 for Wanglang. Here, we propose an effective way to restore gene flow between the two isolated populations.

Key words: Giant panda, microsatellite variation, genetic differentiation, habitat fragmentation, Minshan A habitat.

INTRODUCTION

The giant panda (*Ailuropoda melanoleuca*) is endemic to the mountains of west-central China and is one of the most endangered species in the world (Schaller et al., 1985; Hu, 2001). Historically, this species was widely distributed in most of the lowland subtropical evergreen forests from Zhoukoudian, near Beijing to southern China and into northern Myanmar, northern Vietnam, Laos and Thailand (Wen and He, 1981; Zhu and Long, 1983; Hu, 2001; Zhang et al., 2007). However, it is now restricted to six isolated mountain ranges along the eastern edge of the Tibetan Plateau with likely noT more than 1600 individuals reported in the latest survey (Hu, 2001; Lü et al., 2001; State Forestry Administration, 2006). The range has been fragmented into more than 30 isolated habitats and the population divided into an estimated 24 groups, most with less than 50 individuals (O'Brien et al., 1994; Loucks et al., 2001).

Habitat fragmentation caused by human activities is one of the major threats to the long-term persistence of many species (Young and Clark, 2000; Goossens et al., 2005). Potentially negative effects of fragmentation include the simple reduction of habitat area, modifications of the physical environment and increased isolation of local populations (Primack, 1993). Small and isolated endangered species populations may experience genetic erosion and be more susceptible to demographic and

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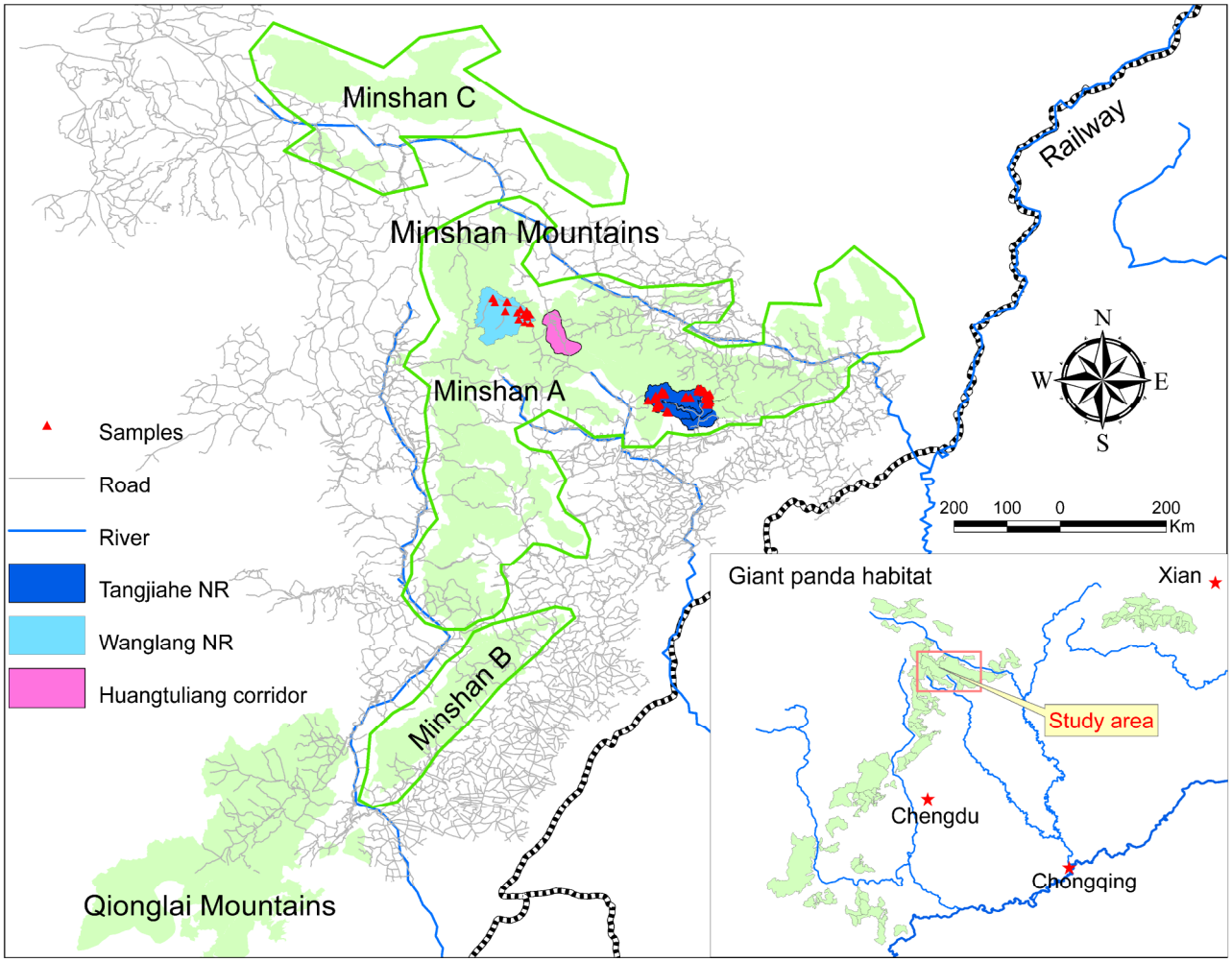

Figure 1. Study areas, with locations of individual giant pandas (*A. melanoleuca*) and road map in the Minshan A region. Inset shows the current giant panda distribution in China.

environmental variation than large populations (Lande,1988; Packer et al., 1991; Pimm and Raven, 2000). It is very difficult for small isolated populations to maintain long-term survival even though excellent habitat or few human disturbances occur (Pimm and Raven, 2000; Loucks et al., 2001; Zhang et al., 2002; Yang et al., 2007). Currently, although a series of steps has been carried out to expand panda habitats and prompt the linkages between the protected areas (Soule et al., 1986; Xiao et al., 2008), the ecological degradation of panda habitats continues to worsen (Liu et al., 2001; Lü et al., 2001; Xiao et al., 2008).

The Minshan region is an area with one of the largest numbers of wild giant pandas (Schaller et al., 1985; Hu, 2001; State Forestry Administration, 2006). However, the Minshan panda habitat is split into 3 strips which are named Minshan A, B and C habitat owing to major road construction (Figure 1). Of the three isolated habitats in the Minshan region, Minshan A habitat is the largest one. But it has been further segmented into three small and isolated habitats in the past 60 years (Hu, 2001; State Forestry Administration, 2006). Both Tangjiahe Nature Reserve (NR) and Wanglang NR are the most critical reserves in the Minshan A habitat, but the two reserves are separated by the Pingwu-Jiuzhai road.

Understanding the genetic structure of populations can provide insight into endangered causes of the species and devising effective conservation strategies (Jarne and Lagoda, 1996; Beaumont and Bruford, 1999; Groombridge et al., 2000; Zhang et al., 2007; Hu et al., 2010). In previous studies, various kinds of molecular genetic were used to assess the giant panda's genetic diversity patterns and examine its evolutionary process (Zhang and Su, 1997; Lü et al., 2001; Zhan et al., 2007; Zhang et al., 2007; He et al., 2008). Recently, research indicates that microsatellite markers can be successfully amplified from fecal samples and could be used to accurately identify individuals, census the population and evaluate population genetic status (Bellemain et al., 2005; Zhan et al., 2006; Zhang et al., 2007; Cronin et al., 2009).

Table 1. Allelic diversity of 10 microsatellite loci for the two giant panda (*A. melanoleuca*) populations (Tangjiahe NR and Wanglang NR), including locus name, number of individuals genotyped for each population (N), numbers of alleles for each population (A), allelic richness for each population (*AR*), numbers of private alleles for each population (*Pr*).

Locus	Total			Tangjiahe				Wanglang			
	N	*A*	*AR*	*N*	*A*	*AR*	*Pr*	*N*	*A*	*AR*	*Pr*
Ame14	54	7	5.528	40	7	5.614	2	14	5	4.680	_
Ame15	58	2	1.999	40	2	2.000	_	18	2	1.988	_
Ame19	54	8	4.351	41	6	4.054	3	13	5	4.308	2
Ame21	49	12	7.877	39	8	6.286	4	10	8	8.000	4
Ame25	50	11	6.799	34	8	5.530	2	16	9	7.322	3
Panda-05	49	7	5.486	36	6	5.305	3	13	4	3.768	1
Panda-22	54	5	3.990	42	4	3.237	1	12	4	3.976	1
Panda-44	46	6	4.066	31	5	4.118	2	15	4	3.333	1
gp001	59	5	4.759	40	5	4.521	_	19	5	4.557	_
gp901	52	8	5.735	41	6	4.535	4	11	4	3.909	2
Total	53	71	5.059	38	57	4.520	21	14	50	4.584	14

Although, Minshan A habitat may be threatened by habitat loss and fragmentation, there have been few efforts to evaluate the effects of habitat fragmentation on population genetic structure of giant pandas. In this study, 10 microsatellite DNA markers were used to determine the amount and distribution of genetic variability present in the two critical populations in Tangjiahe NR and Wanglang NR. Our results will help managers to establish an effective conservation and management strategy for the *in-situ* populations in the Minshan Mountains.

MATERIALS AND METHODS

Sample collection

All non-invasive samples (N = 321) were collected from the wild giant pandas living in two reserves in Minshan Mountains: Tangjiahe NR (104°E, 32°N; N = 201) and Wanglang NR (104°E, 33°N; N = 120). Most samples were < 15 days old as determined from the status of the outer mucosa layer on the feces (Zhan et al., 2006). Locations where samples were taken were GPS recorded and mapped in Arcview 3.2a (Figure 1). Up to five grams of fecal matter was extracted from the outer layer and stored in ASL buffer (QIAGEN, Inc.). We collected hair samples from rub trees and other natural rub objects and stored in small paper envelopes. We sampled over two periods: 120 fecal samples were collected in Wanglang NR in 2005 and then 201 samples in Tangjiahe NR from 2009 to 2010.

DNA preparation, quality verification and microsatellite genotyping

DNA was extracted from hair samples with QIAamp DNA mini kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol and from fecal samples with the use of the QIAamp stool mini kit (QIAGEN, Inc.) using the manufacturer's instructions. The quality of DNA was verified by polymerase chain reaction (PCR) amplifications using three microsatellite loci: Panda-22, gp901 and Ame-μ21 under previously published conditions (Zhang et al., 1995, 2003; Lü et al., 2001; Shen et al., 2007). Samples which were successfully amplified at least once were considered eligible for further microsatellite analysis.

A total of 10 microsatellite loci (Table 1) designed for giant pandas were amplified to analyse the population genetic structure according to previously published conditions (Zhang et al., 1995, 2003; Lü et al., 2001; Shen et al., 2007). PCR products were separated by capillary electrophoresis using POP4 gel on an ABI 3100 automated sequencer (Applied Biosystem Inc.). Alleles were sized using Genscan-500 (ROX) and the size standard within the GenoTyper analysis software version 3.7 (Applied Biosystems Inc.). To obtain reliable genotypes, the 'multi-tube procedure' was employed to avoid genotyping errors due to potential allelic dropout, because the fecal DNA used in this analysis was very dilute (Taberlet et al., 1996; Pompanon et al., 2005). We used the multitube approach of Zhan et al. (2006) to obtain reliable genotypes.

Data analysis

Molecular markers are very useful tools to identify individuals in conservation biology. However, microsatellites' high mutation rates and variations in amplification stability of primers can lead to genotyping errors due to the low quantities and/or quality of DNA in feces and hair (Taberlet et al., 1996; Waits and Leberg, 2000; Roon et al., 2005; Schwartz et al., 2006). In order to guarantee data efficiency, we used MICRO-CHECHER software (Van Oosterhout et al., 2004) to estimate the presence of genotyping errors such as null alleles, large allele dropout or stuttering null alleles in the data set. In order to minimize the genotyping error, we used the multi-tube genotyping approach (Zhan et al., 2006). Individual identification was performed according to the microsatellite genotype (Zhan et al., 2006, 2007).

Cervus 3.0 (Marshall et al., 1998) was employed to estimate genetic polymorphism for each population as the number of alleles per locus (A), observed heterozygosity (H_O) and expected heterozygosity (H_E). Allelic richness (*AR*) was calculated with the FSTAT 2.9.3 program package (Goudet, 2001) in order to bypass the problem that the observed number of alleles per locus (A) is highly dependent on sample size. Allele frequency and private alleles were analyzed using the software Convert 1.31 (Glaubitz, 2004). The Wilcoxon signed-rank test was used to determine significance. For the same reason, a more powerful Markov chain test of Hardy- Weinberg equilibrium (HWE) and linkage

Table 2. Heterzygosity of 10 microsatellite loci for the two giant panda (*A. melanoleuca*) populations (Tangjiahe NR and Wanglang NR), including locus name, observed heterzygosity (H_O), expected heterzygosity (H_E), Wright's inbreeding coefficient (F_{IS}), and *P* HWE *P* values. * Significantly deviated from Hardy-Weinberg equilibrium (P < 0.01).

Locus	Total				Tangjiahe				Wanglang			
	Ho	***He***	***FIS***	***P***	***Ho***	***He***	***FIS***	***P***	***Ho***	***He***	***FIS***	***P***
Ame14	0.685	0.800	0.145	0.0265	0.725	0.804	0.100	0.0572	0.571	0.667	0.148	0.1530
Ame15	0.310	0.403	0.231	0.1210	0.325	0.453	0.285	0.0721	0.278	0.246	-0.133	0.7273
Ame19	0.722	0.702	-0.029	0.0950	0.829	0.679	-0.224	0.0170	0.385	0.566	0.330	0.0887
Ame21	0.673	0.845	0.204	0.0041*	0.718	0.794	0.097	0.9529	0.500	0.874	0.441	0.0032*
Ame25	0.740	0.827	0.106	0.0513	0.794	0.739	-0.076	0.4700	0.625	0.833	0.256	0.0059*
Panda-05	0.571	0.799	0.287	0.0043*	0.639	0.793	0.197	0.0411	0.385	0.668	0.434	0.0022*
Panda-22	0.630	0.634	0.007	0.1785	0.738	0.569	-0.302	0.0080*	0.250	0.692	0.649	0.0004*
Panda-44	0.630	0.713	0.117	0.0991	0.710	0.692	-0.026	0.8629	0.467	0.579	0.200	0.1933
gp001	0.780	0.772	-0.010	0.4516	0.800	0.728	-0.101	0.1415	0.737	0.666	-0.110	0.8935
gp901	0.596	0.683	0.128	0.0469	0.585	0.643	0.091	0.0447	0.636	0.688	0.079	0.5082
Mean	0.634	0.718	0.118	0.0000*	0.686	0.689	0.005	0.0310	0.483	0.648	0.262	0.0000*

disequilibrium (LD) was conducted with GenePop 3.4 (Raymond and Rousset, 1995) instead of the methods by Cervus. All probability tests were based on the Markov chain method using 1, 000 dememorization steps, 100 batches and 1000 iterations per batch.

We used the FSTAT 2.9.3 program package (Goudet, 2001) to estimate Weir and Cockerham's version of Wright's F-statistics. We also used GDA 1.1 (Lewis and Zaykin, 2002) and Arlequin 3.1 (Excoffier et al., 2005) to calculate F-statistics. We employed Wright's (1978) method to interpret the resultant F_{ST} values in light of suggested qualitative guidelines of F_{ST} values (F_{ST} = 0 to 0.05 indicates little population differentiation, 0.05 to 0.15 indicates moderate differentiation, 0.15 to 0.25 indicates strong differentiation and > 0.25 indicates very strong differentiation). A Bayesian clustering method was used to infer population structure (Structure software, Pritchard et al., 2000). Eight independent runs of K = 1 to 10 were performed at 100000 Markov chain Monte Carlo (MCMC) repetitions with a 100000 burn-in period using no prior information and assuming correlated allele frequencies and admixture. The posterior probability Ln P(D) was then calculated. We identified the optimum K-value by the maximal values of Ln P(D) (the posterior probability of the data for a given K) returned by structure and ΔK-values based on the rate of change in the log probability of data between successive K values (Evanno et al., 2005).

RESULTS

Genetic diversity

A total of 321 samples from two reserves (Tangjiahe NR and Wanglang NR) were collected (Figure 1). The 70 samples (46 from Tangjiahe, 24 from Wanglang) were considered suitable for further microsatellite analysis based on quality verifications. We obtained 70 genotypes from these non-invasive samples and identified 64 unique genotypes (42 in Tangjiahe and 22 in Wanglang). Micro-checker software analysis showed that null alleles might be present, but there was no indication of large allele dropout in the data set. We did not found false alleles (FA) and multiple alleles (MA) in our study. Based on these results, we determined that the data were sufficient for further analysis.

A total of 71 alleles were obtained from the 10 microsatellite loci (Table 1), with a mean allele number per locus of 7.1. Each population sample was found to contain private alleles that were only present in a single population. 21 private alleles occurred in the Tangjiahe population and 14 private alleles in the Wanglang population. Only 36 alleles were shared among the three populations. Allelic richness (*AR*) at each locus ranged from 1.988 to 8.000 alleles in the two populations (Table 1). The Wanglang represented the highest allelic diversity (*AR* = 4.584), followed by the Tangjiahe population (*AR* = 4.520). When compared across all loci, a significant difference was not detected between the Tangjiahe and Wanglang populations (Z= -0.15, P= 0.88).

A large variation in heterozygosity occured in this study (Table 2). The mean observed heterzygosity (H_O) and expected heterzygosity (H_E) was 0.686 (range from 0.325 to 0,829), 0.689 (range from 0.453 to 0.804), respectively for the Tangjiahe population and 0.483 (range from 0.250 to 0.737), 0.648 (range from 0.246 to 0.874) for Wanglang. The difference of H_E was not significant as compared across all loci between the two populations (Z= -1.27, P < 0.20).

HWE heterozygosity, linkage disequilibrium (LD)

The HWE tests showed that only one microsatellite locus (Panda-22) in the Tangjiahe population deviated from HWE (P < 0.01, Table 2), but the other loci were in Hardy-Weinberg equilibrium. All test of HWE (across loci) indicates that Tangjiahe population conformed to Hardy-Weinberg equilibrium. For the Wanglang population, four loci exhibited highly significant departure

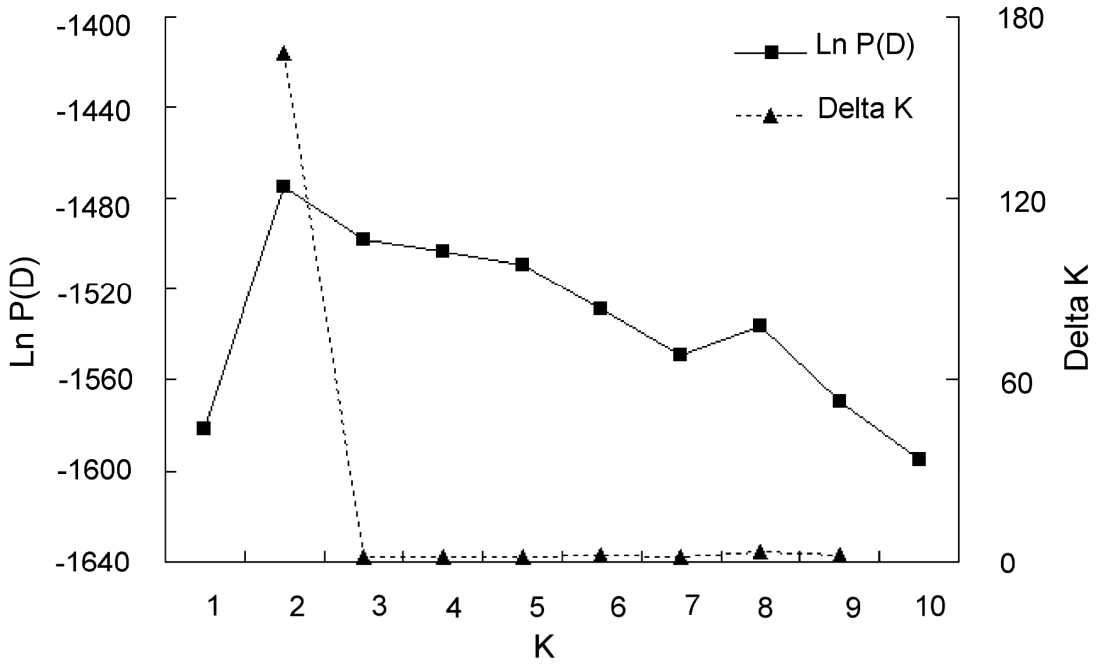

Figure 2. Results from program STRUCTURE analysis of giant pandas (*A. melanoleuca,* N = 64) from Tangjiahe NR and Wanglang NR in Minshan Mountains. Plot displays mean log-likelihood (LnP(D)) and Delta K-values for 10 independent runs of each value of K for K = 1 to 10, indicating that the two sample locations (Tangjiahe NR and Wanglang NR) likely comprise 2 populations.

from Hardy-Weinberg equilibrium due to highly significant heterozygote deficiency (P < 0.01). Overall loci in the Wanglang population did not conform to Hardy-Weinberg equilibrium.

To test whether nonrandom mating was responsible for departure from HWE for these loci, we used F statistics analysis and found a much higher F_{IS} value in the Wanglang (F_{IS} = 0.262, range from -0.133 to 0.649) (Table 2). This indicates a higher inbreeding level for the Wanglang population. In contrast, the level of the Tangjiahe population was low with F_{IS} = 0.005 (range from -0.302 to 0.285). The low F_{IS} value for Tangjiahe reveals a random mating population of giant pandas in Tangjiahe NR. However, following Bonferroni correction, highly significant LD tests did not exist for any pair of loci in the data set.

Population genetic structure

In the genetic differentation analysis, the 95% confidence intervals for F_{ST} values reflected a moderate degree of genetic differentiation (F_{ST} = 0.134, range from 0.071 to 0.172) between the two populations (Tangjiahe and Wanglang). In assignment tests using the Bayesian clustering method in STRUCTURE, 64 unique genotypes were assigned to two clusters (Figure 2). When K = 2, the probability of the data in the Ln probability and ΔK-value were the highest (Ln P(D) = --1475.4, ΔK-value = 168.5). These clusters were largely associated with the population from which they came (Figure 3), although, the data indicated slight gene flow among some of them. Hence, these two populations should be considered genetically distincts. Furthermore, each population contained signature alleles that were only present in a single population (Table 1), which also supported this differentiation.

DISCUSSION

Significant population differentiation of giant pandas in the Minshan A habitat

The Minshan A habitat for giant pandas, is the largest of the three isolated habitats in the Minshan region and is the Minshan biodiversity protection priority zone (Hu et al., 2001; Shen et al., 2009). Lü et al. (2001) and Zhang et al. (2007) reported significant genetic differentiation in almost all pairwise comparisons using microsatellites among the 5 extant mountains populations (Qinling, Minshan, Qionglai, Lianshan and Lesser Xiangling), but they did not further evaluate the effects of fragmentation on population genetic structure of giant pandas within the Minshan A habitat. In our study, we detected significant genetic differentiation of giant pandas between the Tangjiahe and Wanglang populations in the Minshan A habitat (0.05 < F_{ST} < 0.15). This differentiation was supported by the

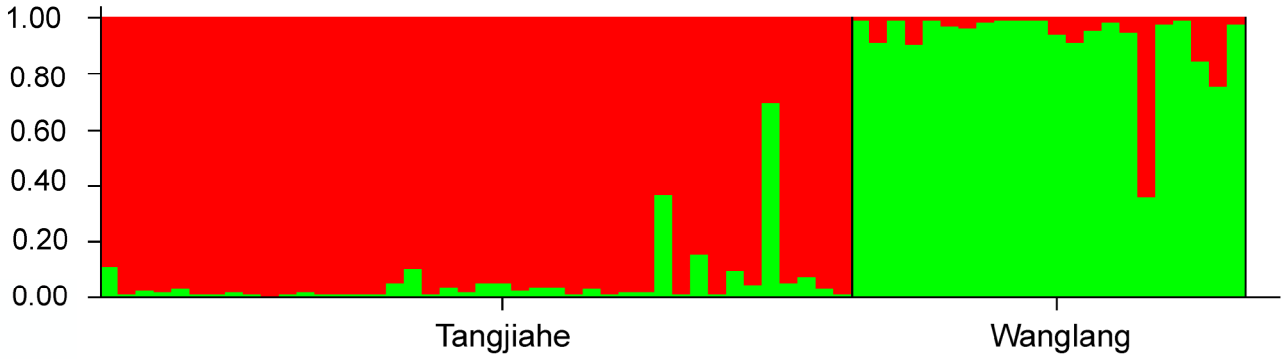

Figure 3. Bayesian cluster analysis of the microsatellite variation for the two giant panda (*A. melanoleuca*) populations (Tangjiahe NR and Wanglang NR). The proportion of ancestry assigned to each of two clusters was plotted by individual. The two colours, green and red, represent two genetic clusters.

assignment tests using the Bayesian clustering method in STRUCTURE (Pritchard et al., 2000). These clusters were largely associated with specific geographical areas, although, the data indicated slight gene flow between the two populations (Figure 2). Their uniqueness was also supported by private alleles (Table1). The mentioned results imply a high level of genetic differentiation between the Tangjiahe and Wanglang populations. Hence, the giant pandas in Tangjiahe NR should be considered a genetically distinct population from the Wanglang population. We speculate that they may have lost contact relatively recently as a result of a complete barrier between adjacent giant panda populations.

In addition, our results indicate that Tangjiahe giant panda population is not threatened by population fragmentation. This conclusion was inconsistent with the results in previous studies (Zhang et al., 2002; Wan et al., 2005). Their results divided giant pandas in Tangjiahe NR into the three subpopulations on the landscape by bamboo bite-size technique in feces (Zhang et al., 2002) and minisatellite DNA based on Southern blotting method (Wan et al., 2005). However, their results seem to greatly contradict with its excellent habitat (Hu, 2005). We argue that these differences in results are mainly due to the application of different studied methods. Bamboo bite-size technique in feces was proven poor at identifying individuals, resulting in a questionable precision of estimates (Zhan et al., 2006). When compared with minisatellite DNA, microsatellites are better genetic markers and widely applied in genetic variation of endangered species (Jarne and Lagoda, 1996; Beaumont and Bruford, 1999; Lü et al., 2001; Zhan et al., 2006; Shen et al., 2009).

Habitat fragmentation and human activities triggered population differentiation

Minshan A habitat covers an area of 6, 200 km^2 and includes 12 reserves (Shen et al., 2009). The Wanglang and Tangjiahe NR are the most important reserves in this area, but they are separated by the Pingwu-Jiuzhai road. Habitat isolation has prevented the mating behavior and obstructed gene exchange between different giant panda groups (Lande, 1988; Packer et al., 1991; Pimm and Raven 2000; Zhang et al., 2007). Huangtuliang on the Pingwu-Jiuzhai road is the most important ecological corridor to link Tangjiahe NR and Wanglang NR (Gong et al., 2003; Yan et al., 2005; State Forestry Administration, 2006). Influenced by road construction and human activities, the habitat area of giant pandas in Huangtuliang strongly has sharply decreased within the past 60 years (Gong et al., 2003; Yan, 2005). Recently, indicators (fecal matter, hair, etc) of giant pandas have rarely been found (Gong et al., 2003; State Forestry Administration, 2006). This indicates that road construction and relevant human activites close to road might be critical threats resulting in population isolation and genetic differentiation of two critical populations in the Minshan A region.

Since the late 1980s, increasingly prosperous tourism has threatened the ecological environment of the Minshan region (Hu, 2001; Gong et al., 2003). The Minshan A region has many famous scenic spots like Jiuzhaigou, Huanglong and Wanglang. The area is segmented into three small and isolated habitats because of Pingwu-jiuzhaigou and Pingwu-Huanglong road constuction and relevant human activites close to road (Gong et al., 2003; State Forestry Administration, 2006). Zhan et al. (2007) reported that apparent genetic divergence was also detected between the Wanglang/Baima and Huanglong populations in the Minshan A habitat due to the road construction. This indicates that the current conservation status of the giant panda habitat in the Minshan A region is not optimistic. Since the Baoji-Chengdu railway was built in the 1950s, more human activities have caused the original distri-bution range of giant pandas in the Minshan region to withdraw 140 km southwards from the northern Minshan and 100 km westwards from the edge area of Longmengshan (Hu, 2001; Gong et al., 2003). This

caused the Minshan A habitat to be reduced by fifty percent within the past 60 years and to become patches and islands.

Populations in small isolated habitat are faced with serious problem such as population fragmentation and genetic erosion more than populations in larger areas (Lande, 1988; Packer et al., 1991; Pimm and Raven, 2000). Habitat fragmentation may accelerate loss of genetic variability due to random genetic drift and a potential increased level of inbreeding in the small remnant populations (Hartl and Clark, 1997; Keller et al., 2004). Changes in allelic diversity may occur faster in isolated populations and cause stronger differentiation between the populations (He et al., 2008). Additionally, the high individual inbreeding coefficients (F_{IS}) in those isolated populations indicate increased levels of homozygosity in wild populations. In our studies, the high individual inbreeding coefficients (F_{IS}) in the Wanglang were tested using microsatellite genotype data. This conclusion was consistent with those of He et al. (2008) and Shen et al. (2009). Fortunately, our results showed that giant pandas among the two studied reserves have preserved a surprisingly high level of genetic diversity and expected heterzygosity (H_E) was 0.689 for Tangjiahe and 0.648 for Wanglang (Table 1). This conclusion was consistent with those of Zhang et al. (2007) and Shen et al. (2009), who concluded that present wild giant panda populations still comprise a rich gene pool.

Implications for conservation

According to the earlier mentioned analysis, we think the core problem of *in situ* conservation in the Minshan A region is population differentiation of giant pandas as a result of habitat loss and fragmentation in the past 60 years (O'Brien et al., 1994; Yan et al., 2005). For small and isolated populations, building giant panda ecological corridors is the most effective way to restore gene flow and increase the persistence of the giant panda population (Hu, 2001; Gong et al., 2003; Yan et al., 2005). With the implementation of the project for giant pandas and their habitats, habitat restoration and other relevant measures have been carried out in the Huangtuliang Corridor (Gong et al., 2003). But now the Pingwu-Jiuzhai road still passes through the core habitat of Huangtuliang. We propose that it is especially essential to build a road tunnel through Huangtuliang and take further effective measures to restore the panda habitat previously destroyed by road construction and human activities as soon as possible. In order to increase the effective protected areas of the reserves, we should enlarge the reserves' area and establish an integrated giant panda nature reserve network, including all the giant panda nature reserves, corridors and surrounding buffer zone in the Minshan A region (Gong et al., 2003). Here, we suggest that Dongyanggou NR should be included in Tangjiahe NR and the Baima community region in Wanglang NR as well.

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REFERENCES

Beaumont MA, Bruford MW (1999). Microsatellites in conservation genetics. In: Goldstein DB, Schlotterer C (eds). Microsatellites: evolution and applications.165–182. Oxford University Press, New York.

Bellemain E, Swenson JE, Tallmon D, Brunberg S, Taberlet P (2005). Estimating population size of elusive animals with DNA from hunter-collected feces: four methods for brown bears. Conserv. Biol., 19: 150– 161.

Cronin MA, Amstrup SC, Talbot S, Sage K, Amstrup KS (2009). Genetic variation and relatedness of polar bears (*Ursus maritimus*) in the Beaufort Sea, Alaska. J. Hered., 100: 681- 690.

Evanno G, Regnaut S, Goudet J (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol., 14: 2611–2620.

Excoffier L, Laval G, Schneider S (2005). Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol. Bioinform. Online, 1: 47–50.

Glaubitz JC (2004). Convert: a user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. Mol. Ecol. Notes, 4: 309–310.

Gong MH, Yu CQ, Chen JS, Yang ZS, Gu XD (2003). Study on the corridors of giant panda. China Forestry Publishing House, Beijing, China.

Goossens B, Chikhi L, Jalil MF, Ancrenaz M, Lackman-ancrenaz I, Mohamed M, Andau P, Bruford MW (2005). Patterns of genetic diversity and migration in increasingly fragmented and declining orang-utan (*Pongo pygmaeus*). Mol. Ecol., 14: 441–456.

Goudet J (2001). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from http:// www.unil.ch/izea/softwares/fstat.html. Updated from Goudet J (1995) FSTAT v-1.2. A computer program to calculate F-statistics. J. Hered, 86: 485–486.

Groombridge JJ, Jones CG, Bruford MW, Nicols RA (2000). Genetic analysis tells of conservation success in the Mauritius kestrel. Nature, 403: 616.

Hartl DL, Clark AG (1997). Principles of Population Genetics, 3rd edn. Sinauer Associates, Sunderland, MA.

He W, Ling L, Shen FJ, Zhang WP, Zhang ZH, Emily K,Yue BS (2008). Genetic diversities of the giant panda (*Ailuropoda melanoleuca*) in Wanglang and Baoxing Nature Reserves. Conserv. Genet., 9: 1541-1546.

Hu JC (2001). Research on the giant panda. Shanghai Publishing House of Science and Technology, Shanghai, China.
Hu JC (2005). A report of the comprehensive survey on Tangjiahe Nature Reserve in Sichuan, China. Sichuan Publishing House Sci. Technol. Chengdu, China.
Hu YB, Zhang XJ, Qi DW, Wei FW (2010). Spatial genetic structure and dispersal of giant pandas on a mountain-range scale. Conserv. Gent., 11: 2145–2155.
Jarne P, Lagoda PJ (1996). Microsatellites, from molecules to populations and back. Trends Ecol. Evol., 11: 424–429.
Keller I, Nentwig W, Largiader CR (2004). Recent habitat fragmentation due to roads can lead to significant genetic differentiation in an abundant flightless ground beetle. Mol. Ecol., 13: 2983–2994.
Lande R (1988). Genetics and demography in biological conservation. Science, 241: 1455–1460.
Lewis PO, Zaykin D (2002). Genetic Data Analysis: computer program for the analysis of allelic data. Version 1.1 . Available: http://lewis.eeb.uconn.edu/lewishome/.
Liu JG, Linderman M, Ouyang ZY, An L,Yang J, Zhang H (2001). Ecological degradation in protected areas: the case of Wolong nature reserve for giant pandas. Science, 292: 98–101.
Loucks CJ, Lu Z, Dinerstein E, Wang H, Olson DM, Zhu CQ, Wang D (2001). Giant pandas in a changing landscape. Science, 294: 1465.
Lü Z, Johnson WE, Menotti-Raymond M, Yuhki N, Martenson JS, Mainka S, Huang SQ, Zhang ZH, Li GH, Pan WS, Mao XR, O'Brien SJ (2001). Patterns of genetic diversity in remaining giant panda populations. Conserv. Biol., 15: 1596–1607.
Marshall TC, Slate J, Kruuk L, Pemberton JM (1998). Statistical confidence for likelihood-based paternity inference in natural populations. Mol. Ecol., 7: 639–655.
O'Brien SJ, Pan WS, Lu Z (1994). Pandas, people and policy. Nature, 369: 179-180.
Packer CA, Pusey E, Rowley H, Gilbert DA, Martenson J, O'Brien SJ (1991). A case study of a population bottleneck: lions of the Ngorongoro Crater. Conserv. Biol., 5: 219–230.
Pimm SL, Raven P (2000). Biodiversity: extinction by numbers. Nature, 403: 843–845.
Pompanon F, Bonin A, Bellemain E, Taberlet P (2005). Genotyping errors: causes, consequences and solutions. Nat. Rev. Genet., 6: 847–859.
Primack RB (1993). Essentials of Conservation Biology. Sinauer Associates Inc, Sunderland, MA.
Pritchard JK, Stephens M, Donnelly P (2000). Inference of population structure using multilocus genotype data. Genetics, 155, 945–959.Software available:http:// pritch.bsd.uchicago.edu/software.
Raymond M, Rousset F (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Hered., 86: 248–249.
Roon DA, Waits LP, Kendall KC (2005). A simulation test of the effectiveness of several methods for error-checking non-invasive genetic data. Anim. Conserv., 8: 203–215.
Schaller GB, Hu JC, Pan WS, Zhu J (1985). The Giant Panda of Wolong. University of Chicago Press, Chicago.
Schwartz MK, Cushman SA, Mckelveyk S, Hayden J, Engkjer C (2006). Detecting genotyping errors and describing American black bear movement in northern Idaho. Ursus, 17: 138–148.
Shen FJ, Watts PC, He W, Zhang ZH, Zhang AJ, Sanderson SJ, Kemp S, Yue BS (2007). Di-, tri- and teranucleotide microsatellite loci for the giant panda, *Ailuropoda melanoleuca*. Mol. Ecol. Notes, 7: 1268–1270.
Shen FJ, Zhang ZH, He W, Yue BS, Zhang AJ, Zhang L, Hou R, Wang CD, Watanabe T. (2009). Microsatellite variability reveals the necessity for genetic input from wild giant pandas (*Ailuropoda melanoleuca*) into the captive population. Mol. Ecol., 18: 1061–1070.
Soule M, Gilpin M, Conway W, Foose T (1986). The millennium ark: how long a voyage, how many staterooms, how many panssengers? Zoo. Biol., 5:.101–113.
State Forestry Administration (2006). The 3rd national survey report on giant panda in China. Science Press, Beijing, China.
Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J (1996). Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic. Acids Res., 24: 3189–3194.
Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004). Software for identifying and correcting genotype errors in microsatellite data. Mol. Ecol. Notes, 4: 535-53.
Waits JL, Leberg PL (2000). Biases associated with population estimation using molecular tagging. Anim. Conserv., 3: 191–199.
Wan QH, Fang SG. LI JG, Zhang LM, Ou WF, Xian FH, Chen WL (2005). A family net of giant pandas in the Tangjiahe Natural Reserve: Assessment of current individual migration. Chinese Sci. Bull., (in Chinese) 50: 1879–1886.
Wen HR, He YH (1981). The distribution of giant panda in Henan, Hubei, Hunan and Sichuan in last five thousands years. J. Southwest Teach. Coll. (in Chinese) 1: 87–93.
Wright S, 1978. Evolution and the genetics of population. In: Variability within and among natural populations, University of Chicago Press, Chicago, Illinois, 5: 694.
Xiao Y, Ou-yang ZY, Zhao JZ, Huang BR, Zhu CQ (2008). An assessment of giant panda (*Ailuropoda melanoleuca*) habitat stressors in Minshan region. Acta Ecol. Sin., (in Chinese) 28: 267–273.
Yan X (2005). Status, challenge and prospect of wild giant pandas. Acta Theriol. Sin., (in Chinese) 25: 402–406.
Yang Z, Hu J, Liu N (2007). The influence of dispersal on the metapopulation viability of giant panda (*Ailuropoda melanoleuca*) in the Minshan Mountains. Acta Zool. Acad. Sci. Hungar., 53: 169–184.
Young AG, Clark GM (2000). Genetics, demography, and viability of fragmented populations. Cambridge University Press, Cambridge, United Kingdom.
Zhang ZJ, Hu JC, Wu H, Hou WR (2002). An analysis on population viability for Giant Panda in Tangjiahe. Acta Ecol. Sin., (in Chinese) 22: 990–998.
Zhan XJ, Li M, Zhang ZJ, Goossens B, Chen YP, Wang HJ, Bruford MW, Wei FW (2006). Molecular censusing doubles giant panda population estimate in a key nature reserve. Curr. Biol., 16: 451–452.
Zhan XJ, Zhang ZJ, Wu H, Goossens B, Li M, Jiang SW, Bruford MW, Wei FW (2007). Molecular analysis of dispersal in giant pandas. Mol. Ecol., 16: 3792–3800.
Zhang BW, Li M, Zhang ZJ, Goossens B, Zhu LF, Zhang SN, Hu JC, Bruford MW, Wei FW (2007). Genetic viability and population history of the giant panda, putting an end to the "evolutionary dead end"?. Mol. Biol. Evol., 24: 1801–1810.
Zhang YP, Su B (1997). Genetic diversity of giant panda. In: Hu, Z., Zhang, Y. (Eds). Genetic diversity of animals and plants in China. 7–18. Zhejiang Publishing House Sci. Technol. Hangzhou, China.
Zhang YP, Wang W, Su B, Ryder OA, Fan ZY, Zhang HM, He TM (1995). The filter and apply of giant panda microsatillite DNA. Zool. Res., (in Chinese) 16: 301–306.
Zhang ZH, Shen FJ, Sun S, David VA, Zhang AJ, O'Brien SJ (2003). Paternity assignment of giant panda by microsatellite genotyping. Hereditas (Beijing) (in Chinese) 25: 504–510.
Zhu J, Long Z (1983). The vicissitudes of the giant panda. Acta Zool. Sin., 29: 93–104.