MOLECULAR ECOLOGY

Molecular Ecology (2011) **20**, 1122–1132 doi: 10.1111/j.1365-294X.2011.04999.x

Significant genetic boundaries and spatial dynamics of giant pandas occupying fragmented habitat across southwest China

LIFENG ZHU,* SHANNING ZHANG,† XIAODONG GU‡ and FUWEN WEI*

**Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, 1-5 Beichenxilu, Beijing 100101, China, †China Wildlife Conservation Association, No 18, Hepingli East Street, Beijing 100714, China, ‡Wildlife Conservation Division, Sichuan Forestry Department, Chengdu, Sichuan 610081, China*

Abstract

Understanding population history and genetic structure are key drivers of ecological research. Here, we studied two highly fragmented and isolated populations (Xiaoxiangling and Daxiangling) of giant pandas (*Ailuropoda melanoleuca*) at the extreme southwestern edge of their distribution. This area also contains the Dadu River, national road 108 and various human infrastructure and development, providing an ideal region in which we can identify the effects of different barriers on animal movements. We used partial mitochondrial control region (mtDNA) and nine microsatellite loci (nuclear DNA) data derived from 192 faecal and one blood sample collected from the wild. We found 136 genotypes corresponding to 53 unique multilocus genotypes and eight unique control region haplotypes (653 bp). Significant genetic boundaries correlated spatially with the Dadu River ($K = 2$). We estimate that a major divergence took place between these populations 26 000 years BP, at around the similar time the rock surface of valley bottom formed in Dadu River. The national road has resulted in further recent population differentiation (Pairwise F_S on mtDNA and nuclear DNA) so that in effect, four smaller sub-populations now exist. Promisingly, we identified two possible first-generation migrants and their migration paths, and recommended the immediate construction of a number of corridors. Fortunately, the Chinese government has accepted our advice and is now planning corridor construction.

Keywords: conservation, genetic boundaries, giant pandas, habitat fragmentation, spatial dynamics

Received 17 May 2010; revision received 22 November 2010; accepted 2 December 2010

Introduction

Understanding population history and genetic structure is key t to drive ecological research (Rockwood 2006). Research using molecular tools based on mitochondrial and nuclear microsatellite markers has shown that natural landscape features such as mountains and rivers can function as genetic boundaries and shape the population structure of animals (Funk *et al.* 2001; Whiteley *et al.* 2004). Anthropogenic landscape features also impact upon genetic structure (Gaines *et al.* 1997) and population dynamics (Nupp & Swhart 1998). For example, roads have lead to population differentiation in a variety of mammals, including bears (Powell *et al.* 1996; Mace *et al.* 1999).

One of China's largest bears, the giant panda (*Ailuropoda melanoleuca*), is regarded as one of the most endangered mammals in the world. Giant pandas are confined to six mountain fragments along the edge of the Tibetan Plateau (Fig. 1). They are elusive with home ranges of between 6 and 14 km^2 and are known to disperse several kilometres (Schaller *et al.* 1985). Habitat destruction continues to impact upon this species. One region in particular, encompassing the Xiaoxiangling and Daxiangling Mountains, contains four subpopula-

Correspondence: Fuwen Wei, Fax: +86 10 64807099;
E-mail: weifw@ioz.ac.cn

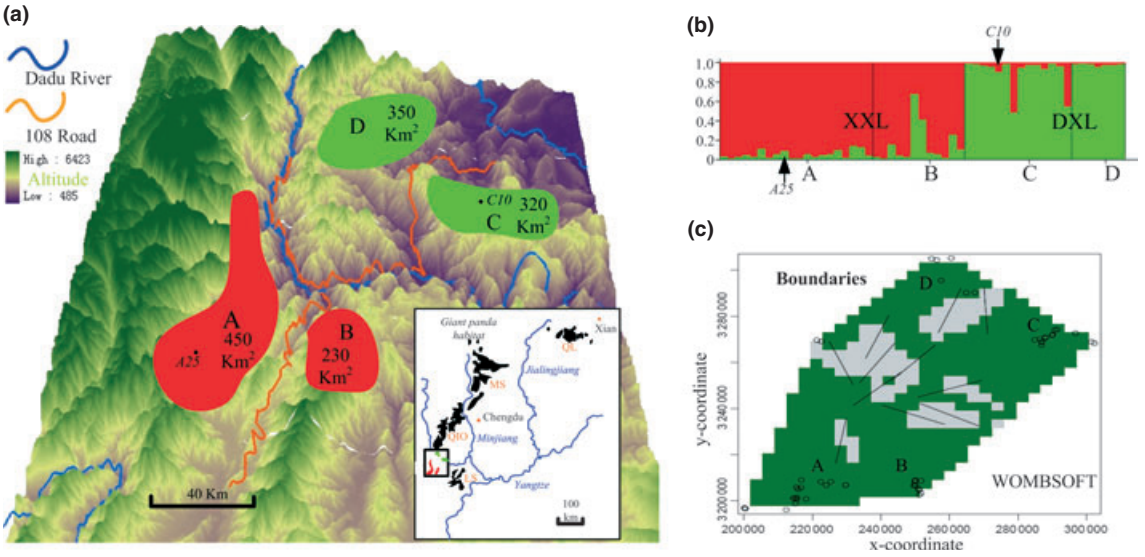

Fig. 1 Study area and population structure in the study region. (a) Study area including the four patches (A: parts of Mianing, Shimian, Jiulong and Luding counties; B: Liziping Nature Reserve; C and D: parts of Yinjing county). The right corner is the current distribution region of giant pandas (QL: Qinling Mountains; MS: Minshan Mountains; QIO: Qionglai Mountains; LS: Liangshan Mountains; the black box is our study region. (b) Genetic clusters ($K = 2$) using STRUCTURE analysis. (c) Genetic boundaries between the Xiaoxiangling and Daxiangling Mountains using WOMBSOFT analysis (the grey region represents the genetic boundary regions).

tions of giant pandas at the southwestern edge of this species' range. These populations are now the most isolated and fragmented within China due to the Dadu River, a major road and human-modified landscape features. The nearest core areas containing giant pandas are over 100 km away at Liangshan and Qionglai.

National road 108 was built in 1938 and effectively dissected habitat in this area into four patches occupied by approximately 60 giant pandas (State Forestry Administration 2006). This road is used by an average of 130 cars per hour and serves as a transportation and communication segue between Sichuan and Yunnan province according to data from the Sichuan Forestry Department. In contrast to this relatively recent landscape feature, the Dadu River has likely affected the movement of giant pandas since its formation millennia ago because its average width is 100 m and mean water flow 895–1200 m^3/s (data from Sichuan Forestry Department). Some studies have investigated population structure, population history and genetic diversity in giant pandas, but none at the extreme edge of their range or in areas so highly fragmented. Lu *et al.* (2001) used mtDNA and nuclear multilocus DNA from Qionglai, Minshan and Qinling populations and found they retained a large amount of genetic diversity and that human activities have posed a more effective migration barrier over the past few thousand years than ancient river systems. Zhang *et al.* (2007) also found high genetic diversity but that genetic differentiation among populations (Qinling, Minshan, Qionglai, Xiaoxiangling and Liangshan) is significant in most cases. Given historic and continued threats to giant pandas, each and every population is now crucial to their survival. Understanding population structure and genetic diversity and factors affecting these is important to the management and conservation of giant pandas. Within the key region comprising Xiaoxiangling and Daxiangling, whether the Dadu River, highway or other human activity has significantly altered population genetic structure and led to differentiation remains unclear. Herein, we integrate data derived from partial mitochondrial control region and nine nuclear microsatellite loci, and ask (i) which factors have influenced population structure, (ii) has gene flow occurred between these populations, and (iii) what, if any, potential corridors exist. This information will aid in the management of these giant panda populations, allow for prioritization of management outcomes in other populations, and also contribute to our knowledge of population structuring for other fragmented mammal species.

Materials and methods

Study region and sample collection

We surveyed approximately 150 zig-zag transect lines across 1350 km^2 of known giant panda habitat from March to October 2005 and have adopted this transect method previously (Zhan *et al.* 2006). Transects commenced at the base of each mountain (about 2200 m a.s.l. in Xiaoxiangling, about 1200 m a.s.l. in Daxiangling) and two people walking 50–100 m apart then

ascended the mountain while criss-crossing the valley or gully. We surveyed every valley and slope within the search zone to an altitude of 3900 m a.s.l (or 2600 m a.s.l in Daxiangling) or when the terrain became too steep to traverse. Surveys were conducted during daylight hours and by experienced observers. The field team comprised 10 members. We collected 142 faecal samples and one blood sample in Xiaoxiangling, and 50 faecal samples in Daxiangling (Fig. 1a). The single blood sample was collected from a rescued animal in Mianning county transferred to Ya'an Bifengxia Captive Panda Research Center in Sichuan for examination. Majority of samples were <2 weeks old as judged by the status of the mucosal outer layer (Zhan *et al.* 2006). We recorded a GPS position for each sample. In the field, up to 5 g of faeces was peeled from the outer layer and stored in 99% ethanol for transport to the laboratory.

Nuclear genetic analysis

DNA extraction, amplification and genetic diversity. DNA was extracted from faeces following standard procedures (e.g. Zhang *et al.* 2006). Eighteen giant panda microsatellite genetic loci (Lu *et al.* 2001) and three redesigned loci (Shen *et al.* 2005) were initially assessed, and nine loci (Ame-05, Ame-10, Ame-13, Ame-15, Ame-16, Ame-26, Ame-22, AFAY161179 and AY161195) were selected for this study on the basis of PCR efficiency, polymorphisms and yield. To obtain reliable genotypes a multi-tube approach (Taberlet *et al.* 1996; Zhan *et al.* 2006) was used as follows: 50 cycles of PCR amplification were carried out simultaneously for up to four loci, with combinations selected based on fragment size, T_m, and fluorescent dye (FAM, TET, or HEX), using the QIAGEN Mutliplex PCR kit according to manufacturer protocol at optimized annealing temperatures (48 °C). Products were resolved using an ABI 377 prism automated sequencer, and analysed using GeneScan v3.1.2 and Genotyper 2.5 (Applied Biosystems). Gender identification was carried out according to previously described methods (Zhan *et al.* 2006). A species-specific sexing primer pair ZX1 was designed to amplify a 210 bp region of the Y chromosome of the giant panda. PCR and cycling conditions were similar to those used for microsatellite amplification. Each sample was amplified three times with ZX1, and products were separated by electrophoresis on a 2.0% agarose gel. A sample was identified as male if at least two experiments showed the 210 bp SRY band and as female if no bands were produced.

Genotyping errors are frequently encountered in noninvasive genetic analysis using faecal samples (Taberlet *et al.* 1996; Taberlet *et al.* 1999) and preselection of samples and rigorous laboratory procedures must be followed to produce accurate genotypes. As part of this process, we conducted mitochondrial DNA analysis for species verification, and our microsatellite genotyping protocol followed the criteria of Taberlet *et al.* (1996). Genotype error rates were estimated using a mathematical approach (Zhan *et al.* 2009). The consistency of genotypes was checked according to standard replication criteria (Taberlet *et al.* 1996; Taberlet *et al.* 1999). Genetic diversity was measured as the mean number of alleles per locus (A), observed (H_O), and expected (H_E) heterozygosities (Nei 1978). Wright's F statistics were estimated according to Weir & Cockerham (1984). Linkage disequilibrium (LD) was estimated across all pairs of loci using the correlation coefficient of Weir (1979). A permutation approach was used to determine which LD values were significant. The above analyses were computed using Arelquin v3 (Excoffier & Schneider 2005). The presence of null alleles, stuttering and small allele dominance was tested using microchecker (Van Oosterhout *et al.* 2004). No evidence was found for null alleles, stuttering or allele dropout in each locus of each population. Hardy–Weinberg equilibrium was tested by GENEPOP 3.4 (Raymond & Rousset 1995). F_{IS} analyses were performed using the GENETIX software (Belkhir *et al.* 1996/1997). Genotypes from different samples were considered to represent the same individual when all alleles at nine loci were identical or if there was only one mismatch for one allele (Bellemain *et al.* 2005; Solberg *et al.* 2006). The software GIMLET was used to calculated the probabilities of identity [P(ID) and P(ID-sibs)] to quantify the efficacy in discriminating the nine loci in combination.

Population size estimation. Following our previous research (Zhu *et al.* 2010), we used the DNA-based mark-recapture software CAPWIRE (Miller *et al.* 2005) to estimate the population size, especially for the Daxiangling Mountains region. Unlike traditional mark-recapture approaches, this method is efficient in dealing with data inferred from multi-observations of individuals within a sampling session. Pandas are mostly solitary and defecate on average 40 times daily (Hu 2001) therefore we chose the ECM (Even Capture Probability Model: assumes similar deposition rates and even sampling effort among individuals) in CAPWIRE, but evaluated these results against an alternative model, TIRM (Two Innate Rates Model: assumes the population includes individuals with two kinds of capture probabilities). We used 1000 bootstrap replicates to produce 95% confidence intervals of the census population size (Nc) under two models.

Detecting genetic cluster and boundaries. First, a Bayesian clustering method was used to detect structure using STRUCTURE (Pritchard *et al.* 2000). Eight independent runs of K = 1–8 were performed at 2 000 000 Markov Chain

Monte Carlo (MCMC) repetitions with a 200 000 burn-in period using no prior information and assuming correlated allele frequencies and admixture. The posterior probability K [P(K|X)] was then calculated and the log-likelihood was used to choose the most likely value for *K*. In addition, the statistic Δ*K*, the second order rate of change in the log probability of the data between successive values of *K*, was also estimated as recommended by Evanno *et al.* (2005). Second, WOMBSOFT (Crida & Manel 2007), one of the non-Bayesian simulation methods, is an R package that analyses individually geo-referenced multilocus genotypes for the inferences of genetic boundaries between populations. The following were our main parameters (i) the grid size was 30 m; (ii) m = 1500 m, the distance from the border where we added individuals based on the home range of giant pandas was between 3 and 10 km^2 (Hu 2001). (iii) h = 5000 m, the bandwidth according to the dispersal distance of giant pandas (Hu 2001). GENELAND is a computer package that allows the use of georeferenced individual multilocus genotypes to infer the number of populations and the spatial location of genetic discontinuities between populations (Guillot *et al.* 2005, 2009).

Detect first-generation migrant individuals and recent migration rate. Geneclass 2.0 uses a suite of likelihood-based statistics in combination with resampling method to calculate probabilities that individuals are first-generation migrants. We used three likelihood-based test statistics to identify migrant individuals: (i) the ratio of *L_home* to the highest likelihood value among all available population samples including the population where the individual was sampled (*L_max*) (Paetkau *et al.* 2004); and (ii) the ratio of *L_home* to the highest likelihood value among all population samples excluding the population where the individual was sampled (*L_max_not_home*). BayesAss, a different approach based on a Bayesian method relying on Markov chain Monte Carlo techniques recently proposed by Wilson & Rannala (2003) was also used to estimate posterior probabilities of a number of population parameters, including recent migration rates over the last several generations, among populations. This method was more akin to the assignment approaches in that it uses multi-locus genotypes as probabilistic indicators of source population and thus produces estimates of recent migration.

Divergence time analysis. The divergence time between the Xiaoxiangling and Daxiangling populations was also estimated by microsatellite loci as the reference to that of mitochondrial sequences. First, the genetic distance $(\delta\mu)^2$ for microsatellite loci, incorporating features of the stepwise mutation model, was used to estimate the divergence time (Goldstein *et al.* 1995). A major advantage of $(\delta\mu)^2$ is that it depends on time and not on population size. $(\delta\mu)^2$ is linear with time (generation unit), having a slope equal to two times the average mutation rate across loci. The generation time of giant pandas is about 5 years (Hu 2001). POPULATION (http://www.bioinformatics.org/groups/?group_id=84) was used to estimate this value. The mutation rate in giant pandas remains unclear so we used conservative mutation rates of microsatellite loci as a reference, such as that of humans (5.6×0^{-4}), in the analysis.

Mitochondrial DNA analysis

The 5′ end of the mitochondrial control region (653 bp) was amplified by polymerase chain reaction (PCR) (35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C) using the following primers: P-tp (5′- CTC CCT AAG ACT CAA GGA AG -3′, forward primer designed in present study) and BEDH (5′- GGG TGA TCT ATA GTG TTA TGT CC -3′, reverse primer). PCR fragments were purified using the E.Z.N.A. Cycle-pure kit (Omega) and sequenced using the P-tp, BEDL225 (5′- ATG TAC ATA CTG TGC TTG GC -3′) and BEDH primers and the BigDye Terminator Sequencing Ready Reaction V3.0 kit (Applied Biosystems), following the manufacturer's instructions, using an ABI 3700 semi-automated DNA analyser.

Genetic diversity. Sequences for each individual and consensus haplotypes were aligned using DNASTAR (Burland 2000) and rechecked by eye. The mitochondrial partial control region sequences were aligned using CLUSTALW (Thompson *et al.* 1994) and visually checked. The unique haplotypes were verified using NETWORK (Bandelt *et al.* 1999). Nucleotide diversity and haplotype diversity (Nei 1987) were estimated using ARLEQUIN version 3 (Excoffier & Schneider 2005).

Phylogenetic analysis. NETWORK (Bandelt *et al.* 1999) was used to reconstruct phylogenetic networks (Median-joining) and infer ancestral types of the partial control region haplotypes of giant pandas combing all published data (Zhang *et al.* 2007). Furthermore, the program Modeltest (Posada & Crandall 1998) was used to determine the model of substitution that best fits these haplotypes, for constructing maximum-likelihood tree by PHYML, a fast maximum likelihood-based phylogenetic inference (Guindon & Gascuel 2003). Likelihood settings from best-fit model (HKY+I+G) selected. MEGA4 (Tamura *et al.* 2007) was used to construct the Neighbor-joining tree (1000 bootstraps). We selected *Canis indica* as the outgroup belong to the same Carnivora.

Isolation-with-migration model of population divergence. Selection violates the assumptions made by the tests implemented in the Isolation with migration analytical (IMa) software (Hey & Nielsen 2007). To test for selection, Tajima's D (Tajima 1989) was calculated for these partial mitochondrial control sequences and significance was assessed using Arlequin. The 'isolation with migration' model implemented by IMa attempts to fit the data to a null model where an ancestral population bifurcates into two strictly allopatric populations 1 and 2. It implements a Markov chain Monte Carlo (MCMC) simulation to estimate the joint posterior probability of six demographic parameters: θ_1 (population size of species 1) and θ_2 (population size of species 2), θ_A (the ancestral species), m_1, m_2, t (divergence time). In our study, θ_1 refers to the Xiaoxiangling population of giant pandas and θ_2 refers to the Daxiangling population, and the m_1 parameter refers to migration from the Daxiangling population into the Xiaoxiangling population, whereas the m_2 parameter refers to migration from the Xiaoxiangling into the Daxiangling. The model of molecular evolution employed was the Hasegawa–Kishino–Yano (HKY; Hasegawa *et al.* 1985) model. Inheritance scalars of 0.25 for mitochondrial sequences were used. Gene flow, population divergence and $h = 4Ne\mu$ were estimated using IMA (Hey & Nielsen 2007) for all the data. Initially, several runs of IMA were conducted with varying prior values for the theta, migration and divergence time parameters. Once optimal priors were identified, and we settled on prior values of $\theta_1 = 10$, $\theta_2 = 10$, $\theta_A = 10$, $m_1 = 10$, $m_2 = 10$, $t = 10$. We then conducted IMA runs with varying run times, using random number seeds for each and a burn-in period of 200 000 steps. In these runs, we analysed our data using the full isolation-with-migration model and conducted the model-selection procedure independently on each run to explore the effects of runtime variation on the selection procedure. The posterior density function of the full isolation-with-migration model is maximized to generate parameter estimates in IMA (Hey & Nielsen 2007) and the model-selection procedure repeats this process for each of 16 reduced models. Thus, log-likelihood ratio (2LLR) tests were performed to test for the statistical significance of various nested models implemented in the IMa software. The 2LLR values should be approximately chi-squared distributed, and thus, the associated probability values were calculated based on this distribution as recommended (Hey & Nielsen 2007).

Results

We collected 192 faecal samples and one blood sample. From these, we obtained 136 genotypes comprising 53 unique genotypes and eight unique mitochondrial haplotypes. Mean genotype error rate per locus was estimated at 0.16%, and whole genotype identification error across nine loci was estimated at 1.4%. Therefore, we could expect at most two incorrect genotypes identified among our sample of 136. We were able to identify individually 20 animals in patch A (11F, 9M), 12 in B (6F, 6M), 14 in C (7F, 7M) and 7 in D (4F, 3M) (Table 1). The even capture probability model estimated 22 (95% CI: 20–26) giant pandas in patch A, 12 (95% CI: 12–12) in patch B, 20 (95% CI: 14–34) in patch C and 10 (95% CI: 7–24) animals in patch D. The two innate rates model instead estimated 25 (95% CI: 20–35) in patch A, 12 (95% CI: 12–15) in patch B, 22 (95% CI: 14–37) in patch C and 11 (95% CI: 7–27) animals in patch D. Under either model, we appear to have sampled more than half of the animals inhabiting this region.

Table 1 Genetic variability observed within populations of giant panda using nine microsatellite loci and mitochondrial control region

	Microsatellite						Mitochondrial				
Region and patch	NA	PA	MNA	H_O	H_E	F_{IS}	NH	PH	H	π	Tajima's D
Xiaoxiangling A:20 (11F, 9M) B:12 (6F, 6M)	41	8	4.556 ±1.499	0.704 ±0.124	0.656 ±0.139	−0.075 (−0.169 to −0.022)	5	3	0.532 ±0.087	0.0020 ±0.0014	−1.2282 NS
Daxiangling C:14 (7F, 7M) D:7 (4F, 3M)	42	9	4.667 ±1.054	0.660 ±0.148	0.634 ±0.109	−0.04318 (−0.146 to 0.004)	5	3	0.747 ±0.052	0.0016 ±0.0012	−0.1889 NS

F and M, female and male; NA and PA, the number of alleles and private alleles; *H*o and *H*e, observed and expected heterozygosity; MNA, mean number of alleles per microsatellite locus; F_{IS} are reported with their 95% confidence intervals in the bracket. NH and PH, the number of haplotypes and private haplotypes. *H*, haplotype diversity ± variance; π, nucleotide diversity ± variance; NS, non-significant.

Table 2 Haplotype distribution in the Xiaoxiangling and Daxiangling populations

Haplotype [111122446 66] [4134407072 33] [2190178683 58]	Accession numbers	Region			
		Xiaoxiangling*		Daxiangling	
		Patch A	Patch B	Patch C	Patch D
†Gh19 TGTCCCTTTA AC	EF100837.1		1		
†Gh20 CA-T...C.G GT	EF100838.1			1	5
†Gh32 CACT...C.. GT	EF100850.1	2		8	
†Gh33 CA-T...C.. G.	EF100851.1			5	
†Gh37 CACT.T.C.. GT	EF100855.1		7		
†Gh38 CACT..CC.. GT	EF100856.1	17	4		1
†Gh39 CACT..CCA. GT	EF100857.1	1			
†Gh40D CA-TT..C.G GT	HQ610997†				1

*Six of 32 individuals' sequences in Xiaoxiangling have been published in the previous research (Zhang *et al*. 2007).
†The new haplotype found in this study.

The mean number of alleles (MNA) was 4.556 (±1.499, SD) for Xiaoxiangling, and 4.667 (±1.054) for Daxiangling. Average *He* was 0.656 (±0.139), and 0.634 (±0.109) for Xiaoxiangling and Daxiangling respectively (Table 1), similar to other populations of giant pandas in the wild (Zhan *et al*. 2006; Zhang *et al*. 2007). Only one pair-loci significant LD value (0.01 significant level) was found in the across samples test, indicating a negligible effect of LD. There was no significant deviation from Hardy–Weinberg equilibrium for any population ($P > 0.05$). After alignment, 653 base pairs of mtDNA control region sequence were analysed from 53 individuals (identified by nine microsatellite loci). A total of 10 variable nucleotide sites, comprising six singleton variable sites and six parsimony informative sites, were used to define eight haplotypes. Gh32 and Gh38 were shared between the Xiaoxiangling and Daxiangling populations. A different dominant haplotype was found for each patch (patch A: Gh38, 85%; B: Gh37, 58%; C: Gh32, 57%; D: Gh20, 71%; Table 2). Xiaoxiangling had a lower relative haplotype diversity than Daxiangling (Table 1).

Genetic differentiation, structuring and boundaries

Microsatellite data showed significant genetic differentiation along national road 108 ($F_{ST} = 0.033$ for patch A and B, $P < 0.01$; $F_{ST} = 0.037$ for C and D, $P < 0.01$, Table 3) and mitochondrial control region data concurred ($F_{ST} = 0.397$ for patch A and B, $P < 0.01$; $F_{ST} = 0.501$ for C and D, $P < 0.01$). Microsatellite pairwise F_{ST} values ranged between 0.033 and 0.107 and were significant (Table 3). F_{ST} values on the same side of the Dadu River were lower (F_{ST}, 0.033–0.037 for microsatellite and 0.397–0.501 for mitochondrial control region, but only based two pairwise values) than from different sides of the river (F_{ST}, 0.062–0.107 for microsatellite and 0.259–0.752 for mitochondrial control region).

Table 3 Pairwise F_{ST} in the Xiaoxiangling and Daxiangling populations

Patch	A	B	C	D
A				
B	0.033*			
C	0.107*	0.062*		
D	0.107*	0.097*	0.037*	

*Significant level after Bonferroni correction ($P < 0.01$).

Xiaoxiangling and Daxiangling were found to comprise two genetic clusters ($K = 2$) (Fig. 1b and Fig. S1, Supporting information) and genetic boundaries were identified between these populations near the Dadu River (Fig. 1c, Fig. S2, Supporting information). Genetic boundaries were also found between Xiaoxiangling and Daxiangling and between patches A and B, and C and D around the 108 national road (Fig. 1c).

Phylogenetic analysis

Four of eight haplotypes (Gh38, Gh39, Gh22 and Gh40) held the tip position in the bootstrap consensus (Fig. 2a). The haplotype network showed that Gh32 is the most extensively distributed haplotype (Fig. 2b). The maximum-likelihood and Neighbor-Joining trees yielded similar information (data not shown). We found that different haplotypes dominated the Xiaoxiangling (Gh37 and 38) and Daxiangling populations (Gh20, 32, 33) and only two haplotypes were shared (Gh32 and Gh38).

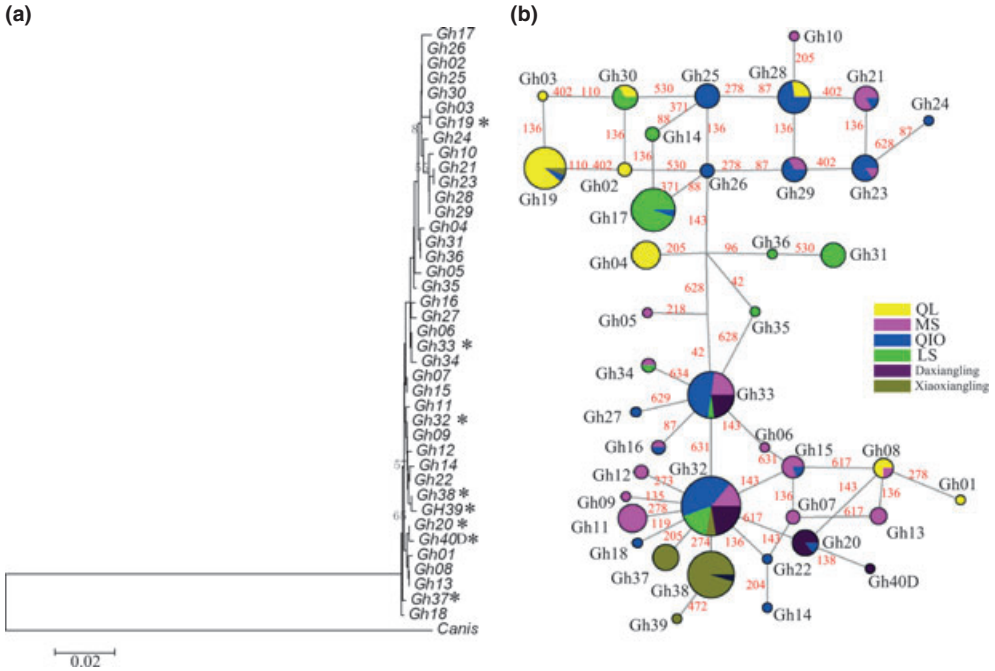

Fig. 2 Phylogenetic neighbour-joining tree (a) and haplotypes median-network (b) among mitochondrial control regions of the Xiaoxiangling and Daxiangling populations under the background of the current giant panda population history. *Haplotypes identified in this study.

Isolation with migration model

The mean posterior probability for Daxiangling to Xiaoxiangling migration rates (scaled for mutation rate) was 2.5726 (Table 4) and for Xiaoxiangling to Daxiangling 2.2701 (Fig. 3 and Table 4). Nonzero rates were inferred for migration in both directions. Population size parameters were used to convert migration parameters into per-generation population migration rates ($M = \theta \times m/2$) and mean values were 2.4440 and 2.1566 migration events per generation for Daxiangling to Xiaoxiangling and Xiaoxiangling to Daxiangling, respectively. Unidirectional Xiaoxiangling and Daxiangling gene flow for either subset was rejected ($P < 0.05$, Table S1, Supporting information). Fifteen of the models were rejected and only one (AAADD) was near the rejected boundary ($P = 0.056$). We found that the ancestral population size was larger (θ_A, 9.7923) than that of Xiaoxiangling or Daxiangling (Table 4 and Fig. S3, Supporting information). The mean divergence time, t (scaled for mutation rate), was 4.8760 (Table 4). We found the mean divergence time was 26 000 BP between Xiaoxiangling and Daxiangling based on the mutation rates of brown bears (*Ursus arctos*, 29.8% per Myr, Saarma *et al.* 2007), a close related species.

The mean $(\delta\mu)^2$ between Xiaoxiangling and Daxiangling was 5.868 (microsatellite data). Microsatellite data revealed that the Daxiangling and Xiaoxiangling populations diverged 26 200 years BP (95% CI: 5000, 52 600).

Migration rates and likely first-generation migrants

Two possible first-generation migrants were identified using the Lh/Lmax-home or Lh/Lmax-not-home ratio and various simulation methods. First, the probability of male A25 sampled in patch A having originated from that patch was just 0.004 [$-\log_{10}$(Lh/Lmax-home) = 2.014] and this male is most likely to have immigrated from patch B [$-\log_{10}$(L) = 9.943]. Second, the probability of female C10 sampled in patch C originating from that

Table 4 Parameter estimates from the empirical data using IMa between Xiaoxiangling and Daxiangling

	θ_1	θ_2	θ_A	m_1	m_2	t
Mean	3.558	7.5668	9.7923	2.5726	2.2701	4.8760
HPD_{90Lo}	0.3074	1.0214	0.0099	0.0050	0.0050	0.2850
HPD_{90Hi}	7.0306	14.5668	17.7004	6.2850	4.8250	9.9950

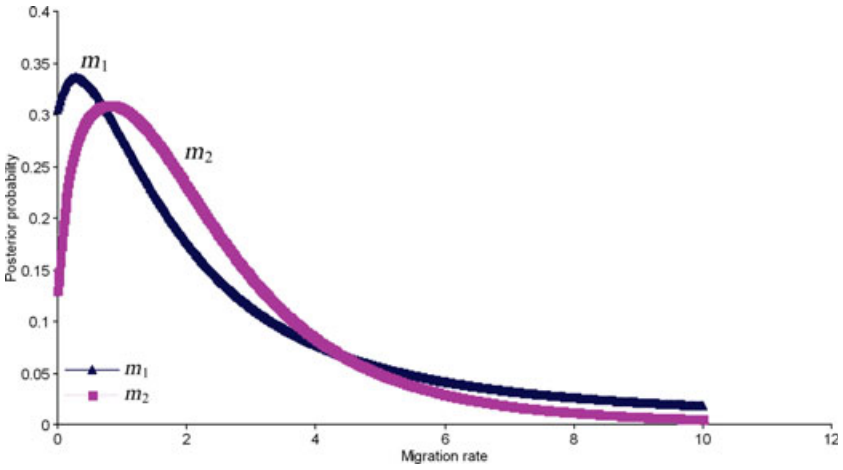

Fig. 3 Posterior probabilities of migration rates determined with IMa. m_1, the migration rate from Daxiangling to Xiaoxiangling; and m_2, the migration rate from Xiaoxiangling to Daxiangling (scaled for mutation rate).

patch was only 0.002 [$-\log_{10}$(Lh/Lmax-home) = 1.911] and is likely to have immigrated from patch D [$-\log_{10}$(L) = 7.539].

We estimated mean immigration rates (last several generations), integrated these into GIS and found that migration is less likely to have occurred across the Dadu River than along it (Fig. 4a). For example, immigration rates, expressed as the proportion of individuals derived from the source population each generation, for Xiaoxiangling to Daxiangling was 0.022 (95% CI: 0.0006, 0.0769), for Daxiangling to Xiaoxiangling was 0.0151 (95% CI: 0.0004, 0.0545), patch A to B was 0.0487 (95% CI: 3.05788e-05, 0.2958), patch B to A was 0.2323 (95% CI: 0.0004, 0.3242), patch C to D was 0.2290 (95% CI: 0.0535, 0.3187), and from D to C was 0.01133 (95% CI: 1.68525e-05, 0.0569).

Discussion

We found that the haplotypes of Xiaoxiangling and Daxiangling populations are closely related to a population at Qionglai, based on haplotypes shared between these groups (mainly Gh32 and Gh33). It also appears from the isolation with migration model (Fig. S3, Supporting information), migration estimates (Table 4), few shared haplotypes and lengthy time since divergence

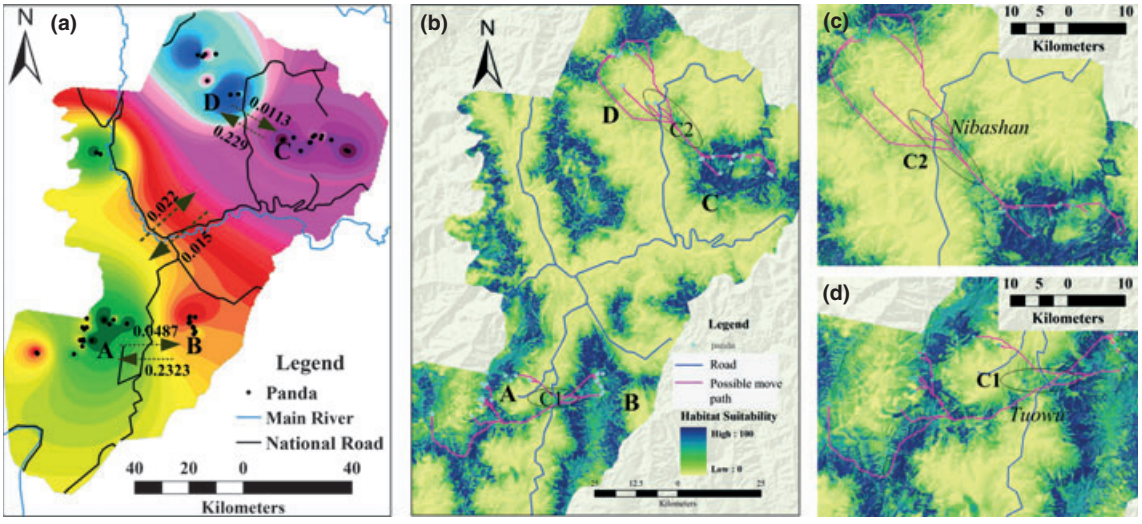

Fig. 4 Recent migration rates between patches and the potential corridor locations. (a) Results of BayesASS integrating individual GIS information under for patch condition. The different colours represent different local genetic origins. (b) Potential corridors identified within Xiaoxiangling and Daxiangling. The background is habitat suitable for giant pandas. (c, d) Detailed positions of two corridors (C1 and C2). C1 in Tuowu village between Patch A and B; C2 in Nibashan region between Patch C and D. Red arrows indicated the possible movement path of giant pandas between patches along national road 108.

that Xiaoxiangling and Daxiangling may have diverged from a single larger ancestral population (Fig. S3, Supporting information).

The Dadu River and population divergence

Several lines of evidence suggest that the Dadu River has played an important role in the divergence of the Xiaoxiangling and Daxiangling populations. First, our estimate of divergence 26 000 years BP is near to the time that the rock surface of valley bottom formed in Dadu River (25 000 years BP) (Wang *et al.* 2006). Second, we identified a single significant genetic boundary between these populations and this boundary is close to the geographic position of the river (Fig. 1c). Last, F_{ST} values for samples from the same side of the Dadu River were lower than for samples from different sides. The total distribution of giant pandas encompasses many large rivers including the Yangtze, Minjiang, Jialingjiang and Dadu. These river systems may have functioned, and continue to function, as barriers to dispersal and movement. Human-driven habitat modification around these rivers is also likely to have contributed to their barrier-like effect (Lu *et al.* 2001), as the Dadu River appears to have done here (Ge 2006). Rivers and associated human infrastructure have been found to impact various species including forest duikers (*Cephalophus grimmia*) (Grubb 2001) and cross river gorillas (*Gorilla gorilla diehli*) (Bergl & Vigilant 2007).

Road 108 and population divergence

National road 108 was found to have impacted population structure among giant pandas in this region since its construction in 1938. Populations along the road appear to have undergone some degree of genetic differentiation. This effect of the road is not as severe as that of the river as mean migration rates were relatively higher for patches separated by the road than patches separated by the Dadu River. The road and associated villages and agriculture still present a barrier to the movement of giant pandas. For example, habitat along the road has been lost or fragmented and resulted in large tracts of hostile landscape that the movement of giant pandas across them are decreased (Xu *et al.* 2006). From Fig. 4b, it is clear just how significant the road and related developments are for giant pandas: for some areas, suitable habitat containing giant pandas are separated by as much as 20 km and beyond known dispersal distances for this species (Schaller *et al.* 1985; Hu 2001). Other studies have shown that highways and development can impose artificial home range boundaries on territorial and reproductive individuals and decrease genetically effective migration (e.g. Riley *et al.* 2006).

Implications for conservation

We posit that the Dadu River is the predominant reason the Xiaoxiangling and Daxiangling populations diverged 26 000 BP. More recently, national road 108 has led to further divergence within these populations. The result is that today this area comprises four sub-populations characterized by different genetic population structures and minimal gene flow. The finding of two possible first generation migrants is promising news for conservationists. The fact these two animals migrated across national road 108 suggests the road is not an absolute barrier to giant panda dispersal and highlights the need for the immediate construction of habitat corridors between patches A and B, and C and D (Fig. 4b–d). Indeed, The Chinese government has now commenced planning these corridors. The first corridor (C1) is around Tuowu village and connects Yele Natural Reserves (core region in patch A) with Liziping Natural Reserves (core region in patch B). The second corridor (C2) is in the Nibashan region and connects Wawushan Natural Reserve (core region in patch C) with the Xinmiao-Sanhe-Jianzheng population (core region in patch D).

Isolation and habitat fragmentation continue to seriously threaten giant pandas. In our study area here for example, patches A–D are over 100 km away from the Liangshan population. Given the amount of population differentiation that has already taken place, and the small number of animals still occupying these patches, the translocation of animals may be required to bolster small populations.

Here, we combined phylogeography, population and landscape genetics to determine population structure in two small and fragmented populations of giant pandas. This study will aid the conservation and management of giant pandas around Daxiangling and Xiaoxiangling, and also those in other areas across China. We have shown how the genetic structure of large mammal populations is affected by natural and anthropogenic factors, and shown this over a fine scale. These findings will help us understand the dynamics and population history of other endangered species in the region such as red pandas (*Ailurus fulgens*), takin (*Budorcas taxicolor*) and other model species around the world.

Acknowledgements

This study was supported by the National Basic Research Program of China (973 Program, 2007CB411600), and National Natural Science Foundation of China (No. 30670329 and 30830020). We thank staff from the Sichuan Forestry Department, Yele Nature Reserve, Lizhiping Nature Reserve and Wawushan Nature Reserve for help during fieldwork. We thank Dr Wang Shichang for assistance with data analysis.

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F.W.'s team at the Institute of Zoology, CAS, Beijing, focuses on animal ecology and conservation genetics in endangered Chinese mammals. L.Z. is interested in the population structure and landscape genetics of endangered species in China. S.Z. is interested in conservation management of endangered species, and X.G. is interested in the landscape ecology and habitat management of large mammals.

Data accessibility

Data deposited at Dryad: doi:10.5061/dryad.8035.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Parameter estimates from the empirical data using IMa between Xiaoxiangling and Daxiangling.

Fig. S1 Genetic clusters from k = 2 to 5 using STRUCTURE.

Fig. S2 Genetic boundary analysis using GENELAND.

Fig. S3 Posterior probabilities of population size determined with IMa.

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