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Genetic differentiation and subspecies development of the giant panda as revealed by DNA fingerprinting

Over the last 100 years giant pandas (*Ailuropoda melanoleuca*) have been separated into six completely isolated mountain ranges. DNA fingerprinting revealed different differentiation patterns in giant pandas, including early-stage, late-stage, recent divergence and recent genetic depression. A separation around 10 000 years ago resulted in highly significant differences in DNA fingerprints and morphological characteristics between Qinling and Sichuan populations. Supported by morphological differences, the genetic data were used to classify the Qinling population as a new subspecies, *A. m. qinlingensis*, while the Sichuan populations were classified into the original subspecies, *A. m. melanoleuca*. Thus, the Qinling population deserves management as a separate unit. In the Sichuan populations, two management units were defined, including Qionglai-Minshan and Daxiangling-Xiaoxiangling-Liangshan. Our data suggest urgent measures are needed to establish green corridors between subpopulations in each mountain range to increase gene flow and genetic variation to ensure long-term survival.

Keywords: DNA fingerprinting / Genetic differentiation / Giant panda / Subspeciation EL 5361

1 Introduction

The giant panda (*Ailuropoda melanoleuca*) is a main member of the giant panda-stegodon fauna group of the Quaternary Period. Although climate changes during the Pleistocene led to the extinction of many members of this group, the giant panda survived. The giant panda was originally distributed over southern and eastern China, extending to northern Burma and northern Vietnam. Today, human activity has resulted in giant pandas being restricted to the isolated Qinling mountain range of Shaanxi Province, and the Minshan, Qionglai, Daxiangling, Xiaoxiangling and Liangshan mountains of Sichuan Province on the edge of the Tibetan plateau (Fig. 1). Much effort has been expended on conservation of the species. Considerable knowledge has been gathered regarding the physiology, ecology, biochemistry, and anatomy of giant pandas, but little is known about the genetic background of wild individuals, which may be an important element of a conservation strategy.

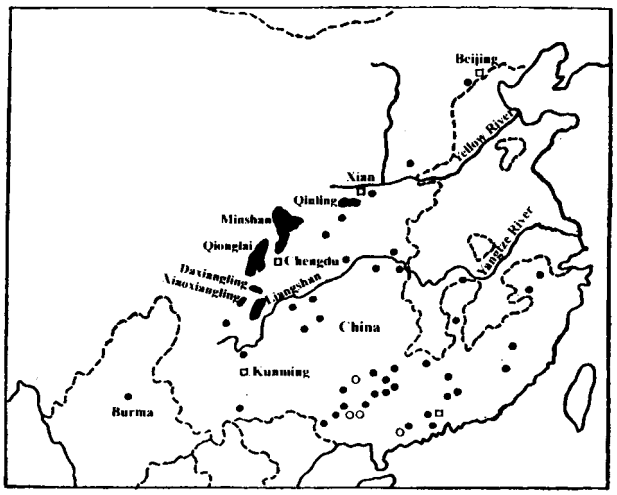

Figure 1. Current and historical distribution of the giant panda. Black areas present distribution; (○) fossil records in the Early Pleistocene; (●) fossil records in the Mid and late Pleistocene; 1 cm corresponds to 400 km; - - - border of Yellow River-Yangtze River lowlands.

In the 1980s the global population of giant pandas was estimated to be ~ 1000 [1]. The small size of the wild population results in inbreeding, causing loss of rare loci and higher homozygosity in descendants. For this reason, appropriate molecular markers and large sample sizes are essential for reliable detection of genetic patterns in pandas. Since the rate of mitochondrial DNA (mtDNA)

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Abbreviations: ANOVA, analysis of variance; **APD**, average percentage difference; **DXL**, Daxiangling; ***F*st**, *F*-statistics; **LSH**, Liangshan; **MSH**, Minshan; **QLA**, Qionglai; **QLI**, Qinling; **SI**, similarity index; **TE**, Tris-EDTA buffer; **XXL**, Xiaoxiangling

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evolution is 5–10 times faster than single-copy nuclear DNA [2], many researchers prefer to use mtDNA as a marker to study genetic variation [3, 4]. However, mtDNA polymorphisms do not provide information about the extent of nuclear gene flow or variability [5]. Furthermore, the rate of mtDNA evolution in some species is extremely low [6], as has been shown for giant pandas [7]. DNA fingerprinting is more suitable for the giant panda because the probes, variable number tandem repeat (VNTR) families, change at a rate of 100–1000 times faster than conventional alleles and can therefore reflect more recent historic events [5, 8]. The development of the new probe gp2000, which produces up to 39 informative loci in giant pandas [9], provides a powerful tool for genetic studies of the species.

Bamboo die-off during 1975–1985 caused the death of several hundred giant pandas from six mountain ranges [10]. Most of these carcasses were preserved by formalin fixation, but the material was not used for genetic study because cross-linking between proteins and DNA makes extraction of high-quality nucleic acid sequences problematic. However, a recent study [11] reported extraction of high-molecular-weight DNA from old formalin-fixed specimens by gradual dehydration and critical point drying, making it possible to use the formalin-fixed panda tissues to study the genetic background of remaining giant panda populations.

2 Materials and methods

2.1 Materials

A total of 126 formalin-fixed specimens from Qinling (QLI, n = 27), Minshan (MSH, n = 31), Qionglai (QLA, n = 24), Daxianling (DXL, n = 14), Xiaoxiangling (XXL, n = 14) and Liangshan (LSH, n = 16) were collected from 17 organizations and museums, including the Foping Natural Reserve, Zoological Institute of Shaanxi Province, Xian Zoo, Department of Forestry of Changxing County of Shaanxi Province, Baishuijiang Natural Reserve, Tangjiahe Natural Reserve, Wolong Natural Reserve, Baoxing Natural Reserve, Animal Specimen Museum of Sichuan Agriculture University, Museum of Sichuan University, Animal Specimen Museum of Sichuan Teacher College, Department of Forestry of Sichuan Province, Chengdu Zoo, Chengdu Research Base for Giant Panda Breeding, Beijing Zoo, Chongqing Zoo and Fuzhou Zoo.

2.2 Sample preparation

Formalin was completely removed from archival tissues by gradual dehydration and critical point drying [11]. Each sample of fixed tissue (0.5 g) was ground in a mortar and pestle. Tissues were incubated in 30% ethanol for 20 min at room temperature and then centrifuged at 3000 × g for 10 min. The procedure was repeated with increasing concentrations of ethanol (+10% graded series) until the pellet was completely dehydrated in 100% ethanol. Pellets were then processed in a critical point drying (HCP-2 Critical Point Dryer; Hitachi, Tokyo, Japan) as described by Fang *et al.* [11]. Genomic DNA was extracted by conventional phenol:chloroform methods [12] and the stringy DNA was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to a final concentration of 0.5 μg/mL.

2.3 DNA fingerprinting

For each sample, 6 μg DNA was digested with 20 U *Hin*f I, and then ethanol precipitated at −20°C. The resultant DNA fragments were dissolved in 6 μL TE buffer and then loaded onto an 0.8% agarose gel. The gels were dried on a vacuum gel dryer (Bio-Rad, Munich, Germany) and hybridized to a [γ-^{32}p]ATP-end (5')-labeled gp2000 probe $(CTCCACCT)_3$. The gp2000 probe end-labelling was done in T4 polynucleotide kinase, according to a previous protocol [13]. Hybridization was performed at 45°C with the probe for 1–2 h in 5 × SSPE (1 × SSPE: 180 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 8.0), 5 × Denhardt's solution (50 × Denhardt's solution: 1% bovine serum albumin, 1% polyvinylpyrrolidone, 1% Ficoll in doubly distilled H_2O), 0.1% SDS, 10 μg/mL sonicated and denatured *Escherichia coli* DNA, and 1–2 × 10^6 cpm/mL of the labelled probe.

2.4 Evaluation and statistics

Genetic variability was assessed by computation of the average percentage difference (APD) using the formula of Gilbert *et al.* [8]. The similarity index (SI) was calculated using the equation in Wetton *et al.* [14]. The relatively unbiased estimate of genetic distance was computed using the formula adapted by Lynch [15] from Nei [16]. *F*-statistics (*F*st) were calculated using the equation modified for DNA fingerprinting by Lynch [17]. Mutation rate was calculated according to the methods of Nürnberg *et al.* [18]. Divergence time was estimated by the formula in Forbes *et al.* [19].

3 Results

3.1 Analyses of DNA banding patterns

Cross-linking between proteins and DNA caused by formalin-fixation resulted in broken DNA, which could potentially have blurred the true genetic variability of the archi-

val samples. However, no evidence of such blurring was found in this study, consistent with the previous results of Fang *et al.* [11]. DNA fingerprints were determined from 126 giant pandas from six mountain ranges (Fig. 2; Tables 1 and 2). Variability of DNA profiles was evaluated by the APD, while inbreeding within populations was indicated by the SI [5, 20]. The intra-mountain range APD varied between populations (Table 1). The two largest populations (MSH and QLA) had the highest APD values (72.23% and 74.36%), whereas samples from the two smallest mountain ranges (DXL and XXL) had the lowest values (60.60% and 63.92%). Comparison with the APD values of 70–90% typically found in outbred vertebrate populations [5] indicates that the level of genetic variability in giant panda populations is relatively low. The MSH and QLA populations had SI values of 0.5536 and 0.5128, respectively, indicating the least inbreeding. DXL and XXL populations had the highest SI values, 0.7881 and 0.7271, respectively, indicating high levels of inbreeding. The QLA population is smaller than the MSH population, but QLA had a better genetic status, suggesting that the effective population size at MSH is smaller than that at QLA. The level of relatedness among the individuals can be evaluated by SI [8], thus the inter-mountain range SI revealed the relatedness between populations. The SI values between QLI and Sichuan populations are smaller than the intra-Sichuan population SI values, indicating an earlier split between the QLI and Sichuan populations (Table 2).

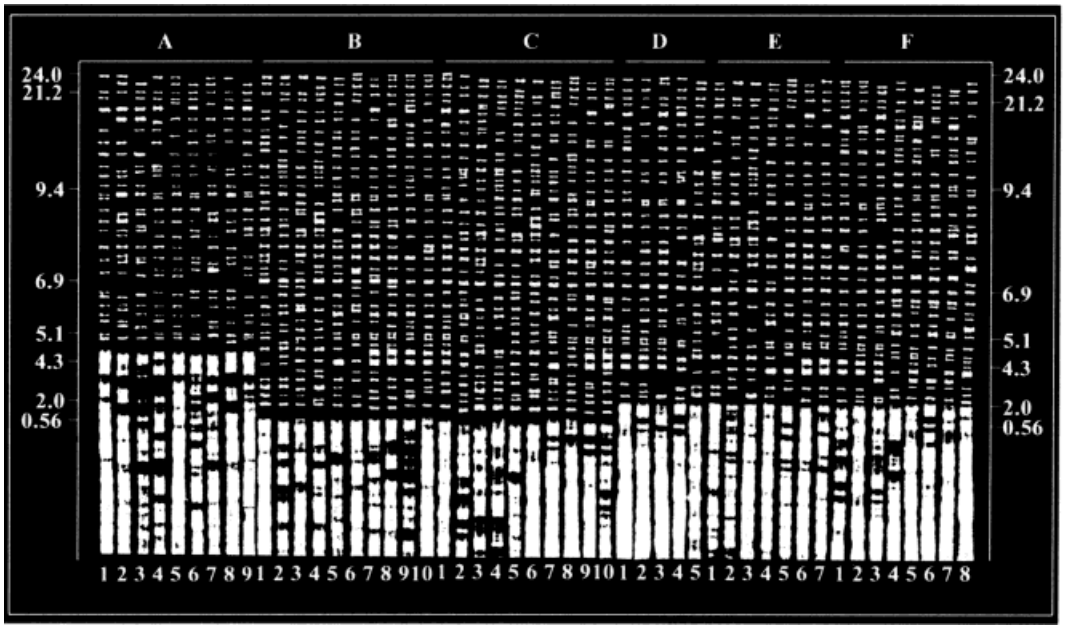

Figure 2. DNA fingerprints of some panda samples from six mountain ranges. (A) Qinling (QLI); (B) Minshan (MSH); (C) Qionglai (QLA); (D) Daxiangling (DXL), (E) Xiaoxiangling (XXL); (F) Liangshan (LSH). Sizes of markers in kb.

Table 1. The genetic diversity parameters for six populations of giant pandas

Parameters	QLI (*n* = 109)	MSH (*n* = 579)	QLA (*n* = 233)	DXL (*n* = 20)	XXL (*n* = 16)	LSH (*n* = 155)
Mean number of bands (*n*)	24.0148	35.6207	36.3341	29.8300	28.2524	33.1435
Range of band distribution (kb)	0.4–24.0	0.1–24.0	0.1–24.0	0.2–24.0	0.2–24.0	0.2–24.0
Sharing band (kb)	21.2	21.2, 6.9	21.2, 6.9	21.2, 6.9	21.2, 6.9	21.2, 6.9
APD	71.28%	72.32%	74.36%	60.60%	63.92%	66.25%
SI	0.5744	0.5536	0.5128	0.7881	0.7217	0.6751

Table 2. SI, genetic distance, *F*st estimates and divergence time between six populations of giant pandas

QLI	MSH	QLA	DXL	XXL	LSH	Location	QLI	MSH	QLA	DXL	XXL	LSH
–	0.9849	0.9918	0.8943	0.8091	0.8769	QLI	–	12 464	12 551	11 317	10 239	11 097
0.2106	–	0.0618	0.3075	0.4068	0.4162	MSH	0.4536[a)]	–	782	3 891	5 148	5 267
0.2013	0.5009	–	0.2917	0.3892	0.4102	QLA	0.4457[a)]	0.0346	–	3 691	4 925	5 191
0.2757	0.4856	0.4749	–	0.3764	0.3832	DXL	0.4879[a)]	0.2930[a)]	0.2649[a)]	–	4 763	4 849
0.2866	0.4208	0.4122	0.5176	–	0.1776	XXL	0.4592[a)]	0.3269[a)]	0.2962[a)]	0.4601[a)]	–	2 247
0.2591	0.4032	0.3904	0.4758	0.5844	–	LSH	0.4621[a)]	0.3211[a)]	0.2946[a)]	0.4301[a)]	0.2906[a)]	–

a) The *F*st estimates indicate highly significant differentiation ($P < 0.01$). Left side: SI (below the diagonal) and the genetic distance (above the diagonal). Right side: *F*st estimate (below the diagonal), and the divergence time (above the diagonal).

The mean number of total bands varied among the populations (Table 1). The MSH and QLA populations had the highest mean number of total bands, and the moderately sized QLI population had the lowest mean number of total bands. The scored bands on the DNA fingerprints ranged from 0.4 to 24.0 kb for the QLI population, 0.1 to 24.0 kb for the QLA and MSH populations, and 0.2 to 24.0 kb for the other three populations. A distinct 21.2 kb restriction fragment was present in all giant panda samples, while another distinct band at 6.9 kb was found in all individuals from the Sichuan populations, but not in the QLI population. In view of so many differences among the six populations, Wright's *F*-statistics were applied to measure population subdivision of the giant panda (Table 2). Estimates of *F*st indicated significant differentiation ($P < 0.01$) between any two populations except between MSH and QLA. Thus, we reconstructed the phylogenetic tree for the six populations.

3.2 Reconstruction of the phylogenetic tree

A phenogram tree was constructed based on the genetic distances shown in Table 2, and another parsimonious tree was constructed on the basis of presence-absence analysis of the restriction fragment data shown in Fig. 3. Not only is the topology of both trees similar (Fig. 4), but both show a strong separation of the QLI population from the Sichuan lineage. From the Sichuan lineage, further divergences occurred resulting in six branches consistent with the current geographical differentiation (Fig. 4a).

3.3 Confirmation of subspecies development

Based on the large genetic differences between the QLI and Sichuan populations, we hypothesized that the giant panda may have differentiated into two subspecies. Key morphometric measurements were taken from 37 adult

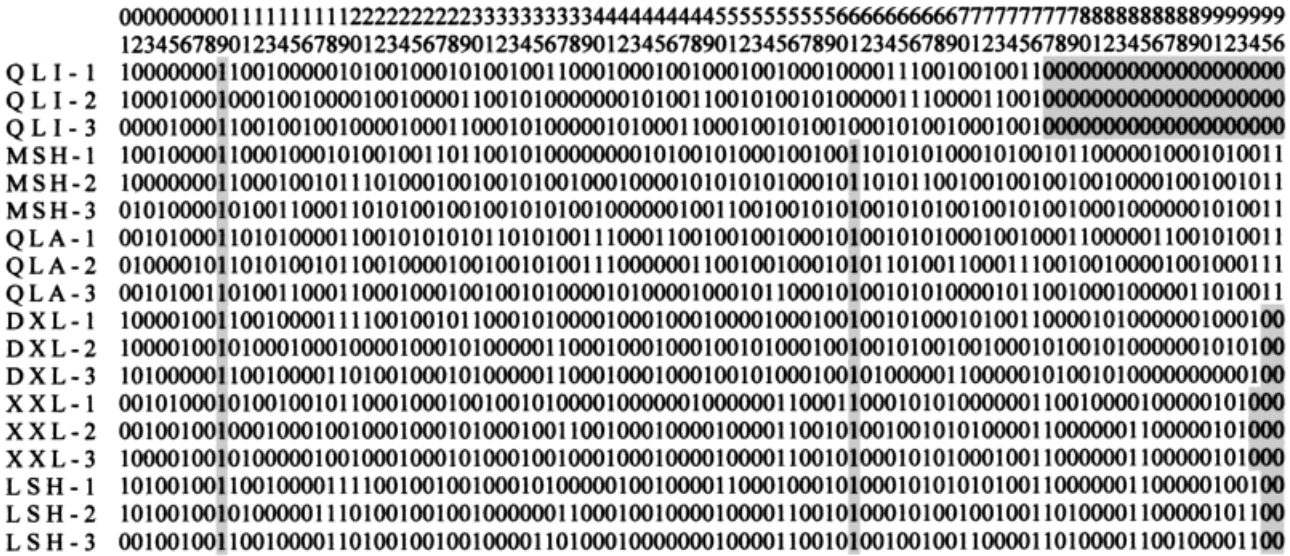

Figure 3. The presence-absence matrix of restriction fragments of 18 individuals from six populations. The shaded numbers are characteristic loci.

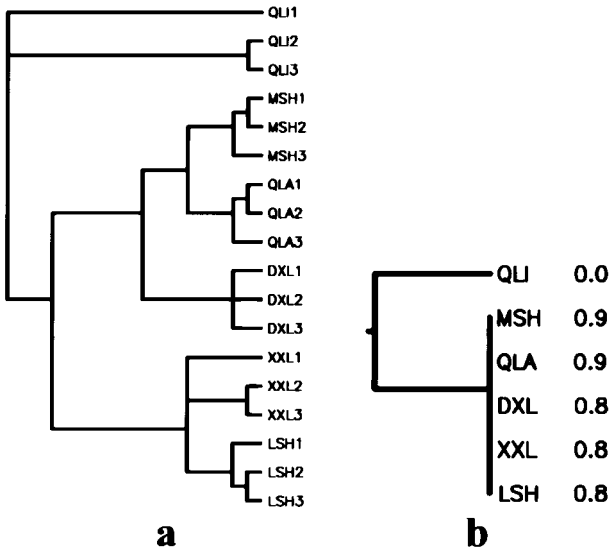

Figure 4. (a) The most parsimonious tree representing the six mountain range pandas, based on the presence-absence matrix shown in Fig. 3. This unrooted tree was generated using the branch-and-bound option of the PAUP program (Version 3.1). (b) Tree based on the genetic distances presented in Table 2. This tree was produced using the UPGMA methods in PHYLIP Version 3.6. The decimals, which are automatically produced by the software based on the phenogram, are not real distances; they show the scale on which the branch lengths will be translated into distances on the output device.

skulls (Table 3) of the QLI (n = 11), MSH (n = 15), QLA (n = 7) and LSH (n = 4) populations. A test for homogeneity of variances was done using the Statistical Package for the Social Sciences (SPSS). No significant differences in the variances existed between the QLI and Sichuan populations (Table 3), and the data were used for analysis of variance (ANOVA). The results revealed very significant differences between the QLI and Sichuan populations, with 7 morphological parameters. The measurements indicate that QLI giant pandas had smaller skulls than Sichuan individuals. The molecular and morphological evidence indicates that two subspecies of the giant panda have formed, *A. m. qinlingensis* and *A. m. melanoleuca*, in QLI and Sichuan, respectively.

3.4 Patterns of genetic differentiation in the giant panda

In small isolated populations, fixation of restriction fragment polymorphisms can outpace the generation of fragment-length variability through recombination [5], causing population-specific or species/subspecies-specific bands [5, 21, 22]. Thus, the 21.2 kb band is specific to the *A. melanoleuca* species of giant pandas, while the 6.9 kb band is specific to the Sichuan subspecies, *A. m. melanoleuca*. This indicates that the entire species underwent a bottleneck that produced the 21.2 kb band. Later, another

Table 3. Comparison of morphological data between Qinling (QLI) and Sichuan (MSH, QLA, DXL, XXL, LSH) giant panda populations

Parameters[a)]	Population	*N*	Mean (mm)	SE (mm)	95% Confid. interval (mm)		Test of HV[b)]	ANOVA	
					Lower bound	Upper bound		*F*-value	Sig.
GLS	Qinling	11	276.7564	3.5041	268.9488	284.5640	0.236	12.340	0.001
	Sichuan	26	295.0604	3.0369	288.8058	301.3150			
CL	Qinling	11	250.5236	2.7339	244.4321	256.6151	0.059	6.985	0.012
	Sichuan	26	262.5273	2.7056	256.9550	268.0997			
BL	Qinling	11	231.6418	2.9929	224.9732	238.3104	0.185	8.247	0.007
	Sichuan	26	243.6504	2.4005	238.7065	248.5943			
PL	Qinling	11	187.3273	2.4080	181.9618	192.6927	0.391	18.125	0.000
	Sichuan	26	201.3869	1.8863	197.5021	205.2718			
OL	Qinling	11	246.4836	3.8086	238.0087	254.9585	0.177	10.747	0.002
	Sichuan	26	265.1835	3.3308	258.3236	272.0433			
CH	Qinling	11	83.1745	1.5137	79.8018	86.5473	0.266	12.234	0.001
	Sichuan	26	90.1685	1.1298	87.8416	92.4953			
LR	Qinling	11	91.8636	1.2094	89.1688	94.5585	0.530	9.954	0.003
	Sichuan	26	97.2685	0.9869	95.2359	99.3010			

a) GLS, greatest length of skull; CL, condylobasal length; BL, basal length; PL, palatal length; OL, occipitonaral length; CH, cranial height; LR, length of rostrum

b) HV, homogeneity of variances, analysis of variance

bottleneck occurred, dividing the population of *A. melanoleuca* into the relatively large QLI population and the smaller Sichuan population. The few founders of the Sichuan population harboring the 6.9 kb band developed into a large population that inhabited the MSH, QLA, DXL, XXL and LSH mountain ranges.

The presence of the diagnostic restriction fragment at 6.9 kb suggests that all Sichuan populations (MSH, QLA, DXL, XXL and LSH) split from QLI at the same time. However, the mean number of total bands and the range of band distribution differ among the six populations, indicating that the mutation rate of each population is different. Calculating the mutation rate of each population is difficult as the materials from each population are very limited. Following analysis of 2074 bands in DNA fingerprints from 64 offspring and 34 parents from the QLI, MSH, QLA and LSH populations, we found two mutant bands (Fang *et al.*, in preparation). Thus, the average mutation rate for the giant panda is 4.82×10^{-4} per generation. Based on the genetic distance (Table 2), the average mutation rate and generation time of the giant panda (12.2 years) [23], each divergence time was approximately computed (Table 2), and the results were found to be consistent with the PAUP maximum parsimony analysis (Fig. 4a).

Small populations result in the loss of bands specific to individuals, while relatively small populations result in a decline in the average number of total bands [24, 25]. We therefore expect a loss of individual-specific bands in each population. MSH, QLA and LSH populations have more bands than the QLI group so the range of band distribution is different, which is also reflected in differences between MSH-QLA and LSH populations (Fig. 2 and Table 1). However, the LSH samples had 4–5 more bands than the DXL and XXL samples, with the range of bands remaining the same. Because there were no more than 20 pandas in the DXL or XXL populations in the 1980s [26], we inferred a decrease in the number of total bands in these two smallest populations. Despite more bands in DXL samples than in XXL samples, the DXL population showed a higher level of similarity compared to the XXL population. Thus, we further inferred that the DXL pandas have lost more bands than XXL pandas over the last few centuries.

The data presented in Table 2 show the presence of three main differentiation periods: 10 000 years ago, from 5000 to 2000 BC, and the past one thousand years. Fossil records indicate that present-day pandas developed during the late Pleistocene, when two glaciation events occurred [27, 28]. The first of those events, 24 000 years ago, caused the first bottleneck, and the few founders harboring the 21.2 kb band developed into the current panda species. The second of those events, 10 000 years ago, resulted in the second bottleneck, splitting the giant panda species into the more primitive Qinling subspecies and the more evolved Sichuan subspecies harboring the 6.9 kb band.

Humans entered the New Stone Age and the Iron Age 5000 and 2500 years ago [26], respectively. Human activity in giant panda habitats appears to have blocked gene flow resulting in the Sichuan subspecies differentiating into the MSH-QLA, DXL, XXL and LSH populations (Table 2). In the past thousand years, human activities such as overexploitation of the environment and the creation of pollution have caused wildlife habitat loss, resulting in many species becoming extinct [29]. A consequence of such activity also appears to be the recent differentiation between the MSH and QLA pandas, and the differences in genetic composition between the DXL and XXL populations.

4 Discussion

Giant pandas currently live in six isolated mountain ranges, and each population is fragmented into smaller isolated subpopulations. Urgent measures are needed to establish green corridors between subpopulations in each mountain range in order to increase gene flow and genetic variation to ensure long-term survival. According to population status and genetic data from the six panda populations, the current study identified three management units, QLI, MSH-QLA and DXL-XXL-LSH.

The total number of pandas in the two smallest populations, DXL and XXL, is less than 40 individuals, so persistence of deleterious recessive genes may result in relatively rapid extinction of these small populations. To avoid this, pandas from these smallest populations should be included in artificial breeding programs. At the same time, green corridors should be established to link the DXL and XXL groups with the closest LSH groups to create a relatively large population. Although the six mountain ranges were completely isolated from each other by construction of the Baocheng Railway and the Chuanzang and Chuandian highways during the 19th century [26], this study revealed that differentiation occurred at three other times – ten thousand years ago, several thousand years ago and several hundred years ago – and that the earliest differentiation resulted in formation of two subspecies. Despite panda habitats being connected, pandas do not migrate between mountains, perhaps reflecting their sensitivity to human activity. This factor should be considered when assessing human activities within reserves.

Among the six populations, QLA has the fastest rate of evolution. Fortunately, the biggest breeding center for giant pandas has been built in the Wolong Reserve of the QLA mountain range. To protect the evolutionary potential of the QLA population, it is essential to link MSH pandas to a larger population. QLI is the population with the lowest mutation rate and has differentiated into a new species. We recommend that management measures applied to the QLA group should also be applied to the QLI population. It is worth noting that captive-bred giant pandas from the QLI and Sichuan populations have been mated and produced hybrid offspring. These hybrids may be at a disadvantage, sometimes even displaying partial reproductive isolation and differences in adapting to different conditions [29]. Consequently, these descendants should be excluded from the breeding population and subspecies hybridization should be avoided in future.

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