Large-scale Genetic Surveys of a main extant population of wild giant panda (*Ailuropoda melanoleuca*), reveals a urgent need of human management

Wanyu Li[1], Chuang Zhou[1], Meiling Cheng[2], Hongmei Tu[1], Guannan Wang[1], Yeming Mao[3], Yaohua Huang[4], Minghua Chen[5], Megan Price[6], Yang Meng[1], Bisong Yue[7], and Xiuyue Zhang[1]

[1]Sichuan University
[2]China Conservation and Research Center for the Giant Panda
[3]Sichuan Heizhugou National Nature Reserve Administration
[4]Sichuan Mabian National Nature Reserve Administration
[5]Sichuan Meigu National Nature Reserve Administration
[6]Sichuan Key Laboratory of Conservation Biology on Endangered Wildlife, College of Life Sciences, Sichuan University, Chengdu 610065, Sichuan, China
[7]Sichuan Key Laboratory of Conservation Biology on Endangered Wildlife

April 16, 2024

Abstract

There are only six isolated living giant panda populations, and a comprehensive understanding their genetic health status is crucial for the conservation of this vulnerable species. Liangshan Mountains is one of the main distribution areas of living giant pandas and is outside the newly established Giant panda national Park. In this study, 971 giant panda fecal samples were collected in the heartland of Liangshan Mountains (Mabian Dafengding Nature Reserve: MB; Meigu Dafengding Nature Reserve: MG; and Heizhugou Nature Reserve: HZG). Microsatellite makers and mitochondrial D-loop sequences were used to estimate population size and genetic diversity. We identified 92 individuals (MB: 27, MG: 22, HZG: 43) from the three reserves. Our results showed that: 1) Genetic diversity of three giant panda populations was medium-low; 2) Quite a few loci deviated significantly from the Hardy-Weinberg equilibrium and almost all these deviated loci showed significant heterozygote deficiencies and inbreeding; 3) Three giant panda populations have substantial genetic differentiation with the most differentiation between MB and the two other populations; 4) a large amount of giant panda feces outside the three reserves were found, implying the existence of protection gap. These results indicated that the giant panda population in Liangshan Mountains is at an risk of genetic decline or extinction given stochastic events and urgent need of human management. This study revealed that high attention should be paid to the protection of these giant panda populations outside the Giant panda national Park, to ensure them survival in their distribution areas.

Large-scale Genetic Surveys of a main extant population of wild giant panda (*Ailuropoda melanoleuca*), reveals a urgent need of human management

Wanyu Li[1,2], Chuang Zhou[1,2], Meiling Cheng[2,3], Hongmei Tu[1], Guannan Wang[1], Yeming Mao[4], Yaohua Huang[5], Minghua Chen[6], Megan Price[1,2], Yang Meng[1,2], Bisong Yue[2], Xiuyue Zhang[1,2*]

[1]Key Laboratory of Bioresources and Ecoenvironment (Ministry of Education), College of Life Sciences, Sichuan University, Chengdu 610064, Sichuan, China

[2]Sichuan Key Laboratory of Conservation Biology on Endangered Wildlife, College of Life Sciences, Sichuan University, Chengdu 610064, Sichuan, China

[3]State Forestry and Grassland Administration Key Laboratory of Conservation Biology for Rare Animals of the Giant Panda State Park, China Conservation and Research Center for the Giant Panda, Dujiangyan 611830, Sichuan, China

[4]Sichuan Heizhugou National Nature Reserve Administration, Ebian 614300, Sichuan,China

[5]Sichuan Mabian National Nature Reserve Administration, Leshan 614600, Sichuan, China

[6]Sichuan Meigu National Nature Reserve Administration, Meigu 616450, Sichuan, China

*Corresponding author: Xiuyue Zhang (zhangxiuyue@scu.edu.cn)

Abstract

There are only six isolated living giant panda populations, and a comprehensive understanding their genetic health status is crucial for the conservation of this vulnerable species. Liangshan Mountains is one of the main distribution areas of living giant pandas and is outside the newly established Giant panda national Park. In this study, 971 giant panda fecal samples were collected in the heartland of Liangshan Mountains (Mabian Dafengding Nature Reserve: MB; Meigu Dafengding Nature Reserve: MG; and Heizhugou Nature Reserve: HZG). Microsatellite makers and mitochondrial D-loop sequences were used to estimate population size and genetic diversity. We identified 92 individuals (MB: 27, MG: 22, HZG: 43) from the three reserves. Our results showed that: 1) Genetic diversity of three giant panda populations was medium-low; 2) Quite a few loci deviated significantly from the Hardy-Weinberg equilibrium and almost all these deviated loci showed significant heterozygote deficiencies and inbreeding; 3) Three giant panda populations have substantial genetic differentiation with the most differentiation between MB and the two other populations; 4) a large amount of giant panda feces outside the three reserves were found, implying the existence of protection gap. These results indicated that the giant panda population in Liangshan Mountains is at an risk of genetic decline or extinction given stochastic events and urgent need of human management. This study revealed that high attention should be paid to the protection of these giant panda populations outside the Giant panda national Park, to ensure them survival in their distribution areas.

Keywords: Liangshan Mountains, *Ailuropoda melanoleuca* , population size, genetic diversity, genetic differentiation, conservation

Introduction

The giant panda (*Ailuropoda melanoleuca*) is a vulnerable species endemic to China. Although China's recent efforts have greatly increased the number and distribution of the wild population, the giant pandas is only distributed in six isolated mountains, namely Qinling Mountains, Minshan Mountains, Qionglai Mountains, Liangshan Mountains, Daxiangling Mountains and Xiaoxiangling Mountains (State Forestry Administration, 2006). The wild population is subject to different degrees of habitat fragmentation at each of the six mountains, and is further divided into more than 30 small populations (O'Brien et al., 1994; Loucks et al., 2001; Lü et al., 2001; Qing, 2016). Therefore, the giant panda is still at a great risk of extinction (Sichuan Provincial Forestry Department, 2015), particularly being vulnerable to stochastic processes. And thus a comprehensive understanding the population size and genetic health status of giant pandas in these region is crucial for the protection decision-making and conservation of this vulnerable species.

The Liangshan Mountains is the southernmost distribution of giant pandas and is located in the transition zone between the southwest edge of the Sichuan basin and the Qinghai Tibet Plateau. The transition zone is within a global biodiversity hotspot, is highly important for the protection of biodiversity in China and is crucial for giant panda protection (Fan et al., 2010). However, the Liangshan Mountains is outside the newly established Giant panda national Park (National Forestry and Grassland Administration (National

Park Administration), 2019) (Figure 1). According to the fourth survey report on giant pandas, there are 124 giant pandas in the Liangshan Mountains and are mainly distributed in Heizhugou, Meigu and Mabian nature reserves (Sichuan Provincial Forestry Department, 2015) (Table 1). These three reserves are located in the heartland of the Liangshan Mountains, and thus are crucial for the protection of giant pandas in the Liangshan Mountains. However, the accurate number, genetic diversity, gene exchange and stable inheritance of panda populations in these key areas remain unclear. Understanding these issues will be vital to the protection of giant pandas in the Liangshan Mountains.

Microsatellite markers have become an important genetic markers in the field of molecular biology (Selkoe and Toonen, 2010) and have been widely used in population surveys (Creel et al., 2003; Piggott et al., 2006; Wang et al., 2016), genetic diversity assessments (Vanhala et al., 1998; Zhang et al., 2007; Shen et al., 2009; Li et al., 2010; Du et al., 2016), and genetic management of populations (Shan et al., 2014). The combined application of microsatellite markers, mitochondrial markers and non-invasive genetic sampling allows giant panda population studies without the risk of capture stress, injury or death, which has contributed greatly to giant pandas conservation in the past 20 years. Consequently, we used microsatellite markers and mitochondrial markers (D-loop) to accurately identify population size and assess the genetic traits of giant pandas in Heizhugou, Meigu and Mabian giant panda populations. We aimed to assess the genetic health status of giant pandas and provide reliable data for establishing genetic archives of giant panda populations and developing the genetic management of giant pandas across the Liangshan Mountains. This is the first extensive genetic survey of giant pandas in the Liangshan Mountains.

Materials and methods

Study area and sample collection

Our study area encompassed Heizhugou, Meigu Dafengding and Mabian Nature Reserves of the Liangshan Mountains (Table 1).

Giant pandas fecal samples were collected by ranger staff during their daily monitoring and patrol work in the reserves. The staff used sterile gloves to collect fresh fecal samples when they detected giant panda activity. Samples were considered fresh based on the color and surface sheen, with dark colored and dull feces being discarded. Each sample was collected in 1-2 copies and stored in a 500 ml sample bottle containing anhydrous ethanol. Spatial coordinates were recorded from the deposition site (e.g., longitude, latitude, elevation) using GPS units and the distribution of samples was mapped as shown in Figure 2, using ArcGIS 10.6 (Price, 2010).

DNA extraction and PCR amplification of mitochondrial D-loop

Fecal DNA was extracted using the kit (Biobase Upure DNA stool kit, Chengdu, China) and nucleic acid purifier (Thermo KlngFisher, USA). Fecal samples collected in the field were soaked in anhydrous ethanol and frozen at -20degC. DNA extraction was undertaken according to the manufacturer's instructions, except for DNA samples being amplified by PCR using the mitochondrial control region primers of the giant panda (Zhang et al., 2007). The total length of the amplification was about 750 bp. The amplification primers were:

P - tp: 5 '- CTCCCTAAGACTCAAGGAAG - 3'

BEDH: 5 '- GGGTGATCTATAGTGTTATGTCC - 3'

PCR amplifications were performed in a 20 μL reaction volume containing 10 μL 2xTaq PCR Pre Mix (+dye), 1μL $MgCl_2$ (25 mmol/L, 0.8 μL BSA (1 mg/ml), 0.8 μL Ptp primer (15 pmol/ L), 0.8 μL BEDH primer (15 pmol/ L), 5 μL template DNA (50 ng/ μL), and 1.6 μL ddH_2O. Amplifications were performed using the following PCR procedure: an initial denaturation step for 5 min at 94°C, followed by 40 cycles of 94°C for 50 s, 55°C annealing for 45 s, 72°C elongation for 50 s and a final elongation for 10 min at 72°C.

Finally, samples were stored at 4°C. PCR conditions were optimized by changing the concentration of Mg^{2+}, annealing temperature and increasing the amount of template DNA.

Selection and amplification of microsatellite markers

Our laboratory has screened giant panda DNA for standardized microsatellite loci and obtained 15 loci that can be effectively applied to giant panda fecal DNA samples (Huang, 2015). We selected seven of these 15 microsatellite loci for population analysis, which were GPL8, GPL29, GPL60, gpz20, gpz47, gpy5 and gpy20 (Table2). PCR amplifications were performed in a 20 μL reaction volume comprising about 10 μL 2xTaq PCR Pre Mix (+dye), 1μL $MgCl_2$ (25 mmol/L), 0.8 μL BSA (1 mg/ml), 0.8 μLF-primer (15 pmol/ L), 0.8 μL R-primer (15 pmol/ L), 5 μL template DNA (50 ng/ μL), and 1.6 μL ddH_2O. Amplifications were performed using the following PCR procedure: an initial denaturation step for 5 min at 94°C, followed by 40 cycles of 94°C for 50 s, 55°C-63°C annealing for 45 s, 72°C elongation for 30 s, and a final elongation for 10 min at 72°C. Finally, samples was stored at 4°C. After the PCR amplification, 5 μL of PCR products from each sample was applied to agarose gel electrophoresis with a concentration of 1.5% to detect whether each sample was successfully amplified. At the end of electrophoresis, the PCR products were stored at 4°C away from light (using tin foil box) for genotyping.

Microsatellite genotyping

Genetic analysis and detection of all samples were undertaken at Chengdu Qingke Zixi Biotechnology Co., Ltd. During genotyping, the amplification products of each fluorescent primer were separately placed in a single lane for electrophoresis. Genotyping of all samples was conducted using ABI 3730 DNA Analyzer. The number of alleles in each sample was determined by using Gene Mapper v4.0. The allele size was determined relative to the intramolecular GS500LIZ.

Data analysis

The results of genotyping data were estimated with Micro-Checker (Van Oosterhout et al., 2004). Individual identification was analyzed using Microsatellite tools (Park et al., 2001). PID and PID (sib) were calculated using Gimlet (Valière, 2010). The Cervus v3.0 (Marshall et al.,2010) was used to calculate Allele number (A), observe Heterozygosity (Ho), expected Heterozygosity (He) and polymorphic information content (PIC). Deviations from the Hardy–Weinberg equilibrium (HWE) and Linkage Disequilibrium (LD) were analyzed using Genepop 3.4 (Raymond and Rousset, 1995). Mitochondrial sequence alignment was performed using MEGA v5.2 (Tamura et al., 2011) and was manually calibrated. DNA ASP v5.10 (Librado and Rozas, 2009) was used to calculate haplotype diversity (h), nucleotide diversity (π) and other genetic diversity indices. Popgene 32 was used to calculate the inbreeding coefficient of population (Yeh, 2000). Population genetic structure analysis was undertaken using STRUCTURE (Pritchard, Stephens & Donnelly, 2000).

Results

DNA extraction and amplification of mitochondrial D-loop region

DNA was extracted from 971 giant panda fecal samples, with a roughly equal sample number from each of the three nature reserves (HZG: 322, MG: 343, MB: 306). We successfully extracted DNA from 731 fecal samples (HZG: 275, MG: 230, MB: 226; Figure S1 for partial electrophoresis).

In this study, the mitochondrial D-loop region was used for genetic diversity assessment, and for fecal DNA quality assessment. The quality of extracted fecal DNA was evaluated by PCR amplification of a 750bp fragment in the mitochondrial D-loop region of the giant panda, to determine the freshness of collected feces. We successfully amplified and sequenced 686 DNA samples equally divided between the three reserves (HZG: 240, MG: 228, MB: 218; Figure S2 for partial electrophoresis).

Individual identification

We found that the minimum number of loci to have greater than 99% confidence of individual identification was six. Analyses determined that six microsatellite combinations were most effective for individual identification for the three populations, calculating PID (sib) 0.00911 and 0.00964 (Table 3; Figure 3). According to Waits et al. (2001), a PID less than 0.01 is required to evaluate population size. PID (sib) avoids errors associated with PID and provides a conservative upper estimate of the number of loci required to identify individuals.

Individual identification using microsatellites determined that the number of giant pandas in the three reserves was 27 (MB), 22 (MG) and 43 (HZG). Two of the reserves' individual numbers were higher than the fourth survey (18 (MB), 22 (MG) and 29 (HZG)). Identification of larger populations in MB and HZG may have two causes, not necessarily mutually exclusive. The first being that different methods were used for our study and the fourth survey. The fourth survey predominantly used the "Distance-Bamboo Stem Fragments Method" adopted in the third survey (Shi et al., 2016). The fourth survey did employ non-invasive DNA quantity survey technology, but it was only used as an auxiliary survey. Therefore, the limitations of the fourth survey may have underestimated the number of giant pandas. Secondly, the population's emigration, immigration, births and deaths will may influence differences in population number estimation. For example, we detected two individuals from Meigu Nature Reserve in Heizhugou Nature Reserve.

Genetic diversity based on microsatellite markers

Seven microsatellite markers were successfully amplified from the three populations. Ninety-six alleles were detected at seven loci in 92 individuals from the three populations. There were 13 common alleles in the three populations. We identified five unique alleles in each of Mabian and Meigu populations (Figure 4). Similarly, these two populations had the same number of rare alleles (9). The numbers of unique and rare alleles in Heizhugou population were 15 and 13, respectively (Figure 4). These rare alleles are at risk of being lost due to inbreeding or genetic drift.

The number of alleles at each locus ranged from 1 to 10. The average number of alleles in Heizhugou population was the largest, followed by Meigu population and Mabian population. The average observed heterozygosity (Ho) of the three populations was 0.632 (MB), 0.598 (MG) and 0.466 (HZG), the average expected heterozygosity (He) was 0.577 (MB), 0.502 (MG) and 0.555 (HZG), and the polymorphic information content (PIC) was 0.514 (MB), 0.441 (MG) and 0.508 (HZG), respectively (Table 4). Therefore, the three populations showed a moderate-low level of genetic diversity. The HWE test results showed that four of the seven microsatellite loci in Mabian population deviated from HWE ($P < 0.01$), while three in Meigu population and two in Heizhugou population (Table 4). A positive mean inbreeding coefficient (Fis) value was found in Heizhugou population (Table 4). High inbreeding coefficient suggests a heterozygote deficiency due to inbreeding. Our results are similar to Guan et al. (2009), who concluded that their observed HWE deviation was due to inbreeding and genetic drift.

Genetic diversity based on mitochondrial control region sequence

We successfully sequenced the mitochondrial D-loops from 85 of the 92 individuals from the three reserves, with sequencing peaks shown in Figure S3. The number of mitochondrial D-loop sequences (n), haplotypes (H), variation sites (s), haplotype diversity (h), and nucleotide diversity (π) of the three populations are summarized alongside other wild and captive populations in Table 5. Compared to other populations, the mitochondrial genetic diversity of giant pandas in these three reserves was significantly lower than in wild populations from Qinling, Minshan and Qionglai Mountains. Mitochondrial genetic diversity of three populations was also lower than captive populations from Wolong, Chengdu and Shaanxi, but higher than Daxiangling and Xiaoxiangling populations.

Geographic isolation and genetic differentiation

According to the distribution map of giant panda fecal samples (Figure 2), feces collected in Heizhugou and Meigu Nature Reserves were often in close proximity to the border between the two reserves. Samples collected in Mabian Nature Reserve were far away from collection sites in Meigu and Heizhugou Nature

Reserves.

The software STRUCTURE (Pritchard, Stephens & Donnelly, 2000) was used to analyze the population genetic structure. Our results showed that when K=2, the value of $^{K}peakedanddecreasedwithincreasingvaluesofK.AsshowninFigure5,thegiantpandasofthreereserveswereclearlydividedin$

The Fst of giant panda population pair-wise comparisons from the three reserves was calculated and measured by GenALEx 6.5 (Peakall and Smouse, 2012). We used the Fst to represent interpopulation differentiation (Zeng, 2014). Studies have shown that if the range of Fst is 0.00-0.05, the genetic differentiation between populations is small and can be ignored. If Fst is between 0.05 and 0.15, there is a moderate degree of differentiation and between 0.15 and 0.25 indicates a high degree of differentiation (Wright, 1972; Liu, 2012). The results showed that there was a significant genetic differentiation between the three giant panda populations, with Fst ranging from 0.0756 to 0.1588 (Table 6). The Mabian population had a significantly higher degree of genetic differentiation with Meigu and Heizhuguo population, while there is a moderate degree of differentiation between Heizhuguo and Meigu population.

Discussion

Genetic health assessment of populations

The protection of species genetic diversity has always been the core of species protection (Frankham, 2005). The evaluation of the genetic diversity within the protected species can allow conservators to predict the probability of population extinction or survival when under stress and to provide an theoretical basis for the effective conservation of population. In this study, the genetic diversity of Mabian (Ho = 0.6324, He = 0.5773), Meigu (Ho = 0.598, He = 0.502) and Heizhugou (Ho = 0.466, He = 0.555) populations were lower than the diversity of Wolong wild population (Ho = 0.644, He = 0.684) and Shaanxi captive population (Ho = 0.610, He = 0.593) (Huang, 2015), but higher than the diversity of the wild Qinling Mountains population (Ho = 0.451, He = 0.439) (Ji, 2014). The D-loop region of mitochondrial DNA (mtDNA) is characterized by high base replacement rate (Yu et al., 2004), which is suitable for analyzing the genetic characteristics of a population. Haplotype diversity (h) and nucleotide diversity (π) are two important indicators to measure the level of population genetic variation. We found that mean h and π values from the three reserves were significantly lower than Qionglai, Qinling and Minshan wild populations, and also lower than Wolong, Chengdu and Shaanxi captive populations, only higher than that of Daxiangling and Xiaoxiangling wild populations (Table 5). Genetic diversity analysis based on microsatellite markers and mitochondrial control region sequences showed that the genetic diversity level of giant pandas in three Liangshan mountains populations was at a medium-low level, and the presences of rare alleles and inbreeding may further reduce their genetic diversity levels. These results show it is necessary to introduce new genetic resource into the three populations or enhance gene exchange between the three populations and/or other populations.

Serious genetic imbalance may lead to the loss of genetic diversity and population decline (Kvist et al., 2015). The Hardy-Weinberg equilibrium is often used as an assessment of genetic balance within a population (Guo et al., 1992). The Hardy-Weinberg equilibrium test results showed that four of the seven microsatellite loci in the Mabian population deviated from the Hardy-Weinberg equilibrium ($P < 0.01$), while three deviated in the Meigu population and two deviated from the Hardy-Weinberg equilibrium in the Heizhuguo population. Almost all loci that deviated from the Hardy-Weinberg equilibrium showed significant heterozygote deficiencies and significant inbreeding. Inbreeding may be the main cause of deviations from the Hardy-Weinberg equilibrium. Our results showed that the three giant panda populations, especially Mabian, are genetically unbalanced and there is the risk of further loss of genetic diversity.

Fecal samples were most frequently collected in roughly two geographical clusters. Feces that were frequently found in Mabian reserve were far away from these collection sites of Feces in Meigu and Heizhugou reserves. This geographical clustering was reflected in genetic structural units and differentiation of the three giant panda populations. The giant pandas in three reserves were clearly divided into two genetic structural units.

The Meigu and Heizhugou populations formed a genetic structural unit, while the Mabian population was a relatively independent genetic structural unit (Figure 5), indicating limited gene exchange between Mabian and two other populations. Further support for observed clustering was the high genetic differentiation of Mabian population (Fst: 0.13320, 0.15880) with the other two populations. The genetic clustering also confirms that the geographical clusters were likely indications of higher panda activity and not an affect of sampling method.

The genetic and geographical clustering of the three populations suggests that there is a barrier preventing genetic exchange between the two areas. Feng (2015) found that suitable habitats were fragmented in central and northern Mabian Nature Reserve. Unsuitable habitats might be caused by deforestation, road construction and livestock invasion (Feng, 2015; Zhao et al., 2017; Zhang et al., 2018). Fragmented suitable habitats and unsuitable habitats could influence the habitat selection and migration of giant panda. These unsuitable habitats are mainly concentrated in the western margin and northern sections of the Mabian Nature Reserve (Feng,2015) and this resulted in giant panda have moved southward. This change might has occurred between the 3rd (1999-2002) and 4th (2011-2014) national panda surveys because the distribution of giant pandas in Mabian moved southward at 4th national panda surveys compared to 3rd surveys (State Forestry Administration, 2006; Sichuan Forestry Department, 2015). This increased geographically distance and potentially barrier effect between Mabian population and other two populations formed the genetic isolation of Mabian population from other two populations.

Conclusively, the level of genetic diversity of three giant panda populations was medium to low, while the genetic diversity of Mabian giant pandas was the lowest. The existence of genetic isolation, a high number of rare alleles, inbreeding and significant deviations from the Hardy-Weinberg equilibrium indicated that these three populations were genetically unstable, and inbreeding may further result in the loss of genetic resources (Wang, 2019).

Genetic management recommendations

Liangshan Mountains is one of the main distribution areas of living giant pandas and belongs to the southernmost distribution of giant pandas. Mabian, Meigu and Heizhugou reserves are located in the heartland of Liangshan Mountains, and are also the core distribution areas of giant pandas in the Liangshan Mountains. The effective protection of the three giant panda populations is crucial for the conservation of all Giant pandas in Liangshan Mountains. The results of our have shown that the three giant panda populations are at risk of decline or extinction given stochastic events, especially the Mabian population. It is therefore urgent to improve each population's genetic status by increasing genetic resources. We recommend two strategies for improving the genetic status of three populations. Firstly, improve genetic diversity of three populations by the introduction of genetically distinct individuals. The China Conservation and Research Center for the Giant Panda and the Chengdu Research Base of the Giant Panda have the largest captive breeding populations of giant pandas in China. These captive populations are genetically stable and distantly related to populations from the Liangshan Mountains (Shan et al., 2014). Therefore, genetic introductions from the two captive breeding populations would increase genetic resources into these core populations of Liangshan Mountains giant panda. However, captive-bred introductions are difficult and require considerable resources and time (Yang et al., 2018), and therefore it should not be the only strategy for the improvement of genetic health.

Our second recommendation for improving the genetic status of the three populations is to increase connectivity and genetic exchange between the two geographically and genetically distinct panda groups. Although significant genetic differentiation between the two groups exists, no significant difference in behavior and morphology has been found. Similarly, there was no evidence that the Mabian population was subject to different geographical or climatic conditions and thus no unique or local adaptation. Therefore, there should be no genetic, behavioral or morphological impediment to breeding and risk of distant hybridization (Frankham, 2010). The fecal sample distribution and population genetics demonstrated there was limited genetic exchange between Mabian and two other populations. However, there is no topographical barrier between the two groups, and the limiting factor is likely from unsuitable habitat and habitat fragmentation

due to disturbance and lack of bamboo vegetation (Feng, 2015; Zhao et al., 2017; Zhang et al., 2018). Consequently, we recommend that suitable habitat and continuity should be rehabilitated and restored. Recent roads should be reforested and prevented from new construction. Human activities, especially grazing and bamboo shoot collection, should be controlled and minimized. Existing natural forest (bamboo) should be protected from further damage and the non-bamboo areas should be rehabilitated. As a priority, restoration should focus on creating corridors through the 'habitat barrier' to increase panda movement as soon as possible and then expand the area and proportion of suitable habitat. Given that pandas begin moving and they breed, there should be an improvement in the genetic health and population stability of giant panda in Liangshan Mountains.

Although Wei et al. (2020) concluded that China's Panda Protection System and nature reserves can achieve the goals of protecting their habitats and biodiversity, and most giant panda nature reserves have been established based on the distribution of giant pandas, however, the gaps, overlapping designations and disparities in management still exist (Xu et al., 2017; Xu et al., 2019). The reserves in the Liangshan Mountains were established early in China's panda protection efforts and zoning was determined roughly according to predicted panda distributions and human activities. However, many factors have changed over time, and pandas have become more flexible in their habitat choices than previously thought (Hull et al., 2014). For example, space utilization by giant pandas gradually expanded outward between the third and fourth surveys. In addition, we found that a large amount of panda activity occurred outside the reserve (Figure 2), indicating gaps in the coverage of the reserve. Although the Giant Panda National Park offers an opportunity to promote more effective management and improve the management system by integrating and expanding the existing reserves, however, Liangshan Mountains is not included in the newly established Giant Panda National Park (National Forestry and Grassland Administration (National Park Administration), 2019). In this case, greater attention should be paid to the protection of the main extant population of wild giant panda. We strongly suggested that the scope of nature reserves in the Liangshan Mountains should be adjusted, by integrating surrounding suitable habitats into the reserve, better protect giant panda habitats, restore degraded habitat, increase gene exchange between populations, and ensure the population stability of giant pandas in Liangshan Mountains.

In conclusion, giant panda populations in Liangshan Mountains had medium-low genetic diversity, with a high number of rare alleles, significant heterozygote deficiencies and inbreeding. Three populations clustered into two geographically and genetically distinct groupings, with the Mabian population being separated from the other two by a large tract of unsuitable habitat. The giant panda population in Liangshan Mountains is genetically unstable and at risk of decline or extinction given stochastic events. It is therefore recommended that connectivity between populations be re-established by improving habitat quality and continuity, and genetic health be enhanced by the introduction of captive-bred distantly related individuals. These changes could be incorporated into the updated conservation plans for the Liangshan Mountains. Our study revealed that high attention should be paid to the protection of these giant panda populations outside the Giant panda national Park, to ensure them survival in their distribution areas, and can serve as a reference for the genetic management of Giant panda populations in other distribution areas and some key conservation species in China and world.

Acknowledgments

We are grateful to Prof. Jianghong Ran´s comments for our manuscript and the staff of the three reserves for collecting samples. This work was supported by the National Natural Science Foundation of China (31770574).

Author contributions

L. wrote the manuscript. W.L., M.C., H.T., and G.W. performed data analysis. C.Z., M.C., H.T., and G.W. performed experiments. Y.M., Y.H., M.C., amd Y.M. were responsible for collecting the samples. X.Z., B.Y., and M.P. revised the manuscript. X.Z. and B.Y. designed and supervised the study.

Declaration of interests

The authors declare no competing interests.

Data accessibility

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Benefit-sharing

We consulted the local residents and manager of the nature reserve who provided biological protection status, and hired the staff of the nature reserve to to help with genetic diversity assessments, including the collection of giant panda fecal samples. The contributions of all individuals to the research are described in the METHODS and ACKNOWLEDGEMENTS, and a research report has been provided to the relevant management departments. The research addresses a priority concern that a urgent human management and high attention should be paid to the protection of these giant panda populations outside the Giant panda national Park, to ensure them survival in their distribution areas. Lastly, as described above, all data have been shared with the broader public via appropriate biological databases.

Figure 1 The relative position of the study area

Figure 2 Sampling locations (lower-left panel)and identified individuals of giant pandas in the study area

Reference

Creel, S., Spong, G., Sands, J. L., Rotella, J., Zeigle, J., Joe, L., Murphy, K. M., & Smith, D. (2003). Population size estimation in Yellowstone wolves with error-prone noninvasive microsatellite genotypes. *Molecular Ecology* , *12* (7), 2003-2009.

doi:10.1046/j.1365-294X.2003.01868.x

Pritchard, J. K., Stephens, M. J., & Donnelly, P. J. (2000). Inference of population structure using multilocus genotype data. *Genetics* ,*155* (2), 945-959.

doi: 10.1093/genetics/155.2.945

Du, Y., Zou, X., Xu, Y., Guo, X., Li, S., Zhang, X., ... & Guo, S. (2016). Microsatellite loci analysis reveals post-bottleneck recovery of genetic diversity in the Tibetan antelope. *Scientific Reports* ,*6* : 35501. doi: 10.1038/srep35501

Fan, L. Q., Dong, L., Zhang, S. L., Ran, J.H., & Yue, B. S. (2010). Landscape Pattern of Giant Panda Habitat in the Liangshan Mountains, Sichuan, China. *Chinese Journal of Applied and Environmental Biology* , *16* (2), 179-184.

doi: 10.3724/SP.J.1145.2010.00179

Feng, Z. R. (2015). Assessment on the suitability of giant panda's habitat in Mabian Dafengding Nature Reserve. *Northeast Forestry University* .

Frankham, R. (2005). Genetics and extinction. *Biological Conservation* , *126* (2), 131-140. doi: 10.1016/j.biocon.2005.05.002

Frankham, R. (2010). Challenges and opportunities of genetic approaches to biological conservation. *Biological Conservation* , *143* , 1919-1927.

doi: 10.1016/j.biocon.2010.05.011

Guan, T. L., Zeng, B., Peng, Q. K., Yue, B. S., & Zou, F. D. (2009). Microsatellite analysis of the genetic structure of captive forest musk deer populations and its implication for conservation-sciencedirect.*Biochemical Systematics & Ecology* , *37* (3), 166-173. doi: 10.1016/j.bse.2009.04.001

Guo, S. W., & Thompson, E. A. (1992). Performing the Exact Test of Hardy-Weinberg Proportion for Multiple Alleles. *Biometrics* ,*48* (2), 361-372. doi: 10.2307/2532296

Huang, J. (2015). The development and the applications of universal marker system based on microsatellites of giant panda (*Ailuropoda melanoleuca*). *Sichuan University* .

Hull, V., Roloff, G., Zhang, J., Liu, W., Zhou, S., Huang, J., ... & Liu, J. (2014). A synthesis of giant panda habitat selection.*Ursus* , *25* (2), 148-162.

doi: 10.2192/URSUS-D-13-00011.1

Ji, J. F. (2014). Microsatellite Analysis about Genetic Diversity of Giant Panda in Liangshan and Qinling Mountains. *Guangxi Normal University* .

doi: 10.7666/d.Y2585076

Kvist, L., Giralt, D., Valera, F., Hoi, H., & Lovaszi, P. (2015). Population decline is accompanied by loss of genetic diversity in the Lesser Grey Shrike *lanius minor* . *Ibis* , *153* (1), 98-109. doi: 10.1111/j.1474-919X.2010.01091.x

Li, Y. Z., Xu, X., Shen, F. J., Zhang, W. P., Zhang, Z. H., Hou, R., & Yue, B. S. (2010). Development of new tetranucleotide microsatellite loci and assessment of genetic variation of giant panda in two largest giant panda captive breeding populations. *Journal of Zoology* ,*282* (1), 39-46. doi: 10.1111/j.1469-7998.2010.00707.x

Librado, P., & Rozas, J. (2009) Dnasp v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* , *25* (11): 1451-1452.

Liu, H. B., Wang, Z., Liu, J., Ma, L. Y., Wang, S. Q., Tang, Y., & Pang, X. M. (2012). Genetic Diversity and Genetic Structure of Siberian Apricot Populations in the Yan Mountains. *Scientia Silvae Sinicae* , *48* (8), 68-74.

doi: 10.1080/02724634.2012.652324

Loucks, C. J., Lu, Z., Dinerstein, E., Wang, H., Olson, D. M., Zhu, C., & Wang, D. (2001). Giant pandas in a changing landscape.*Science* , *294* (5546), 1465.

doi: 10.1126/science.1064710

Lü, Z., Johnson, W. E., Menotti-Raymond, M., Yuhki, N., Martenson, J. S., Mainka, S., ... & O'Brien, S. J. (2001). Patterns of genetic diversity in remaining giant panda populations. *Conservation Biology* , *15* (6), 1596-1607.

doi: 10.1046/j.1523-1739.2001.00086.x

Marshall, T.C., Slate, J., Kruuk, L., & Pemberton, J.M. (2010). Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular. Ecology* , *7* (5), 639-655.

doi: 10.1046/j.1365-294x.1998.00374.x

National Forestry and Grassland Administration (National Park Administration), (2019). Master plan for Giant Panda National Park.

O'Brien, S. J., Pan, W. S., & Lu, Z. (1994). Pandas, people and policy.*Nature* , *369* (19), 179-180.doi: 10.1038/369179a0

Park, S. D. E. (2001). Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection. *University of Dublin* .

Peakall, R., & Smouse, P. E. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research an update.*Bioinformatics* , *28* (19), 2537-2539. doi: 10.1093/bioinformatics/bts460

Piggott, M. P., Banks, S. C., Stone, N., Banffy, C., & Taylor, A. C. (2006). Estimating population size of endangered brush-tailed rock-wallaby (*Petrogale penicillata*) colonies using faecal DNA.*Molecular Ecology* , *15* (1): 81-91.

doi: 10.1111/j.1365-294X.2005.02783.x

Price, M. (2015). Mastering ArcGIS. *WCB/McGraw-Hill* .

Qing, J. (2016). Study on the habitat fragmentation and designing corridors of giant panda in Sichuan Province. *China West Normal University* .

doi: CNKI:CDMD:2.1016.748842

Raymond, M., & Rousset, F. (1995). Genepop (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* , *86* (3), 248-249.

doi: 10.1046/j.1420-9101.1995.8030385.x

Selkoe, K. A., & Toonen, R. J. (2010). Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers.*Ecology Letters* , *9* (5), 615-629.

doi: 10.1111/j.1461-0248.2006.00889.x

Shan, L., Hu, Y., Zhu, L., Yan, L., Wang, C., Li, D., ... & Wei, F. (2014). Large-scale genetic survey provides insights into the captive management and reintroduction of giant pandas. *Molecular Biology and Evolution* , *31* (10), 2663-2671.

doi: 10.1093/molbev/msu210

Shen, F. J., Zhang, Z. H., He, W., Yue, B. S., Zhang, A., Zhang, L., ... & Watanabe, T. (2009). Microsatellite variability reveals the necessity for genetic input from wild giant pandas (*Ailuropoda melanoleuca*) into the captive population. *Molecular Ecology* , *18* (6), 1061-1070. doi: 10.1111/j.1365-294X.2009.04086.x

Shi, X. W., Zhang, J. D., & Ouyang, Z. Y. (2016). Research progress on population investigation methods for wild giant panda. *Acta Ecologica Sinica* , *36* (23): 7528-7537.

Sichuan Provincial Forestry Department. (2015). The Pandas of Sichuan: The Fourth Survey Report on Giant Panda in Sichuan Province.*Sichuan Science and Technology Press* .

State Forestry Administration. (2006). The Thrid National Survey Report on Giant Panda in China. *Beijing: Science Press* .

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods.*Molecular Biology Evolution* , *28* (10), 2731-2739. doi: 10.1093/molbev/msr121

Valiere, N. (2010). GIMLET: a computer program for analyzing genetic individual identification data. *Molecular Ecology Notes* ,*2* (3), 377-379.

doi: 10.1046/j.1471-8286.2002.00228.x

Van-Oosterhout, C., Hutchinson, W. F., Wills, D. P. M., & Shipley, P. (2004). Micro-checker software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes ,4* (3), 535-538.

doi: 10.1111/j.1471-8286.2004.00684.x

Vanhala, T., Tuiskula-Haavisto, M., Elo, K., Vilkki, J., & Mki-Tanila, A. (1998). Evaluation of genetic variability and genetic distances between eight chicken lines using microsatellite markers. *Poultry Science* , *77* (6), 783-790.

doi: 10.1093/ps/77.6.783

Waits, L. P., Luikart, G., & Taberlet, P. (2001). Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. *Molecular Ecology* , *10* : 249-256. doi: 10.1046/j.1365-294X.2001.01185.x

Wang, D., Hu, Y. B., Ma, T. X., Nie, Y. G., Xie, Y., & Wei, F. W. (2016). Noninvasive genetics provides insights into the population size and genetic diversity of an Amur tiger population in China.*Integrative Zoology* , *11* (1), 16-24.

doi: 10.1111/1749-4877.12176

Wang, E. Z. (2019). Silver carp genetic diversity from Four Famous Domestic Fishes origin field revealed by Mitochondrial DNA D-loop sequence. South-Central University for Nationalities.

Wei, W., Swaisgood, R. R., Pilfold, N. W., Owen, M. A., Dai, Q., Wei, F., ... & Gu, X. (2020). Assessing the effectiveness of china's panda protection system. *Current Biology* , *30* (7), 1280-1286. doi: 10.1016/j.cub.2020.01.062

Wright, S. (1972). Evolution and the genetics of populations. a treatise in four volumes. volume 4. variability within and among natural populations. *Journal of Biosocial Science* , *4* (2), 253-256. doi: 10.1017/S0021932000008543

Xu, W., Pimm, S. L., Du, A., Su, Y., Fan, X., An, L., ... & Ouyang, Z. (2019). Transforming protected area management in China. *Trends in Ecology & Evolution* , *34* (9), 762–766. doi: 10.1016/j.tree.2019.05.009

Xu, W., Xiao, Y., Zhang, J., Yang, W., Zhang, L., Hull, V., ... & Polasky, S. (2017). Strengthening protected areas for biodiversity and ecosystem services in China.*Proceedings of the National Academy of Sciences of the United States of Americ* a, *114* (7), 1601–1606. doi: 10.1073/pnas.1620503114

Yang, B. (2013). The giant panda (*Ailuropoda melanoleuca*) mitochondrial genome research and genetic diversity analysis of the captive population. Sichuan Agricultural University.

Yang, Z. S., Gu, X. D., Nie, Y. G., Huang, F., Huang, Y., Dai, Q., ... & Wei, F. W. (2018). Reintroduction of the giant panda into the wild: a good start suggests a bright future. *Biological Conservation ,217* , 181-186. doi: 10.1016/j.biocon.2017.08.012

Yeh, F. C., Yang, R., Boyle, T. J., Ye, Z., & Xiyan, J. M. (2000). Popgene32, Microsoft windows based freeware for population genetic analysis, version 1.32. Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Alberta, Canada.

Yu, L., Li, Q. W., Ryder, O. A., & Zhang, Y. P. (2004). Phylogeny of the bears (Ursidae) based on nuclear and mitochondrial genes. *Molecular Phylogenetics & Evolution* , *32* (2), 480-494. doi: 10.1016/j.ympev.2004.02.015

Zhang, B. W., Li, M., Zhang, Z. J., Goossens, B., Zhu, L. F., Zhang, S. N., ... & Wei, F. W. (2007). Genetic viability and population history of the giant panda, putting an end to the "evolutionary dead end"?*Molecular Biology & Evolution* , *24* (8), 1801-1810. doi: 10.1093/molbev/msm099

Zhang, Y. K., Wu, Y. J., Zhang, Q. Y., Ran, J. H., & Price, M. L. (2018). Distribution of a giant panda population influenced by land cover. *The Journal of Wildlife Management* , *82* (6), 1199-1209. doi: 10.1002/jwmg.21477

Zhao, C., Yue, B. S., Ran, J. H., Moermond, T., Hou, N., Yang, X. Y., & Gu, X. D. (2017). Relationship between human disturbance and Endangered giant panda *Ailuropoda melanoleuca* habitat use in the Daxiangling Mountains. *Oryx* , *51* , 146-152. doi: 10.1017/S0030605315000800

Hosted file

`Table 1 .docx` available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management

Hosted file

`Table 2.docx` available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management

Hosted file

`Table 3.docx` available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management

Hosted file

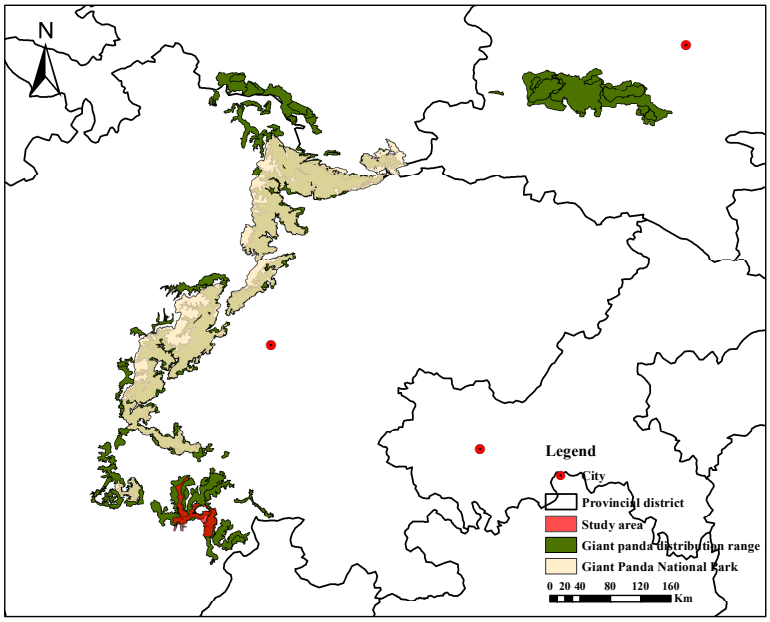
`Table 4.docx` available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management

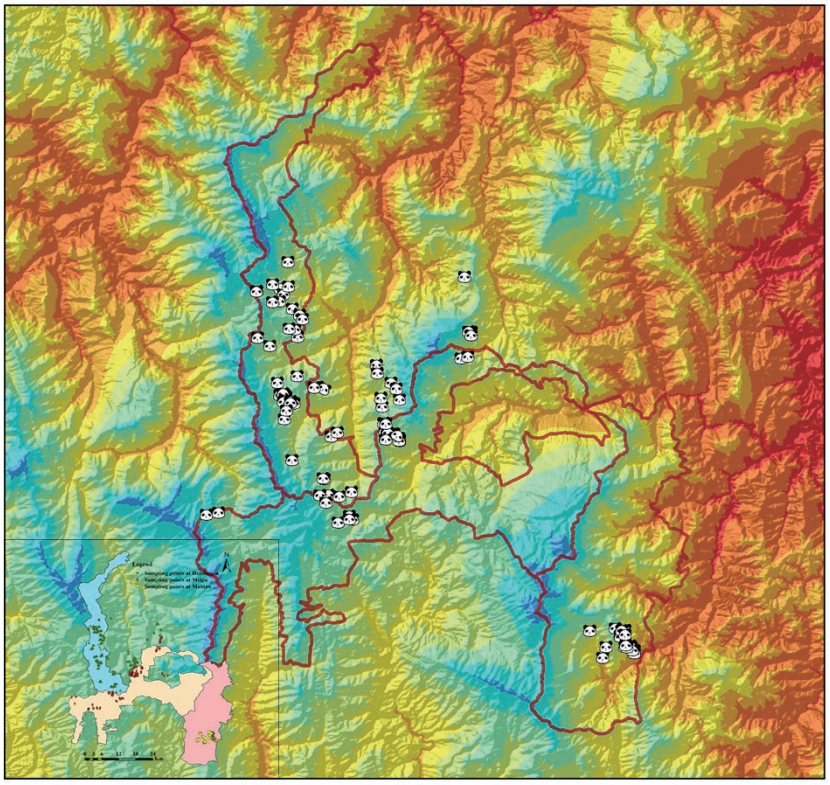
Hosted file

`Table 5 .docx` available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management

Hosted file

`Table 6 .docx` available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management





Hosted file

Figure 3.docx available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management

Hosted file

Figure 4.docx available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management

Hosted file

Figure 5.docx available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management

Hosted file

Figure S1.docx available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management

Hosted file

Figure S2.docx available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management

Hosted file

Figure S3.docx available at https://authorea.com/users/471826/articles/713159-large-scale-genetic-surveys-of-a-main-extant-population-of-wild-giant-panda-ailuropoda-melanoleuca-reveals-a-urgent-need-of-human-management