Large-Scale Genetic Survey Provides Insights into the Captive Management and Reintroduction of Giant Pandas

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Abstract

The captive genetic management of threatened species strives to preserve genetic diversity and avoid inbreeding to ensure populations remain available, healthy, and viable for future reintroduction. Determining and responding to the genetic status of captive populations is therefore paramount to these programs. Here, we genotyped 19 microsatellite loci for 240 captive giant pandas (*Ailuropoda melanoleuca*) (~64% of the captive population) from four breeding centers, Wolong (WL), Chengdu (CD), Louguantai (LGT), and Beijing (BJ), and analyzed 655 bp of mitochondrial DNA control region sequence for 220 of these animals. High levels of genetic diversity and low levels of inbreeding were estimated in the breeding centers, indicating that the captive population is genetically healthy and deliberate further genetic input from wild animals is unnecessary. However, the LGT population faces a higher risk of inbreeding, and significant genetic structure was detected among breeding centers, with LGT–CD and WL–BJ clustering separately. Based on these findings, we highlight that: 1) the LGT population should be managed as an independent captive population to resemble the genetic distinctness of their Qinling Mountain origins; 2) exchange between CD and WL should be encouraged because of similar wild founder sources; 3) the selection of captive individuals for reintroduction should consider their geographic origin, genetic background, and genetic contribution to wild populations; and 4) combining our molecular genetic data with existing pedigree data will better guide giant panda breeding and further reduce inbreeding into the future.

Key words: **giant panda, genetic diversity, inbreeding, captive management, reintroduction.**

Introduction

In the past four decades, many species have experienced rapid population decline and habitat loss due to ongoing threats (e.g., climate change, human activities, invasive species, and disease) (Butchart et al. 2010; Wei et al. 2014). In 2002, world leaders committed "to achieve by 2010 a significant reduction of the current rate of biodiversity loss" (Secretariat of the Convention on Biological Diversity 2003). Despite failing to meet this target (Gilbert 2009; Walpole et al. 2009; Butchart et al. 2010), conservation efforts have been effective and gains have been achieved. For example, some extinctions have been prevented (Rodrigues 2006), population trajectories have been improved (Donald et al. 2007), and the rate of biodiversity loss has been reduced by 20% (Hoffmann et al. 2010). Hoffmann et al. (2010) found that 64 species on the IUCN Red List underwent an improvement in status due to conservation action. In particular, 13 of these 64 species showed improvements directly due to captive breeding (Balmford et al. 2011; Conde et al. 2011a, 2011b; Hoffmann et al. 2011) and this practice is now critical to the survival of a growing number of species (Olney et al. 1994; Frankham et al. 2010; Scott et al. 2010; Conde et al. 2011a). Captive breeding has led to several well-known conservation success stories, including the Przewalski's horse (*Equus ferus przewalskii*), black-footed ferret (*Mustela nigripes*), California condor (*Gymnogyps californianus*), Arabian oryx (*Oryx leucoryx*), European bison (*Bison bonasus*), and red wolf (*Canis rufus*) (Gusset and Dick 2012).

The giant panda (*A. melanoleuca*) is one of the most endangered mammals in the world, and is a well-known and iconic conservation species (Wei et al. 2012). Because of large-scale habitat loss and fragmentation, poaching, zoo collecting, and massive bamboo flowering events, over 1,000 giant pandas were killed, died or removed from the wild before 1988 when the Chinese government enacted the "Wildlife Protection Law" and established significant in situ and ex situ conservation programs (Zhu et al. 2013). It is estimated that about 2,500 giant pandas (Zhan et al. 2006) confined to six fragmented mountain ranges on the eastern edge of the Tibetan Plateau (Schaller et al. 1985; Hu 2001) remain in the wild. Sixty-five giant panda reserves have been established across its range; and three large breeding centers, the

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Chengdu Research Base of Giant Panda Breeding (hereafter CD), China Research and Conservation Center for the Giant Panda at Wolong (hereafter WL), and Shaanxi (Louguantai) Rescue and Breeding Center for Rare Wildlife (hereafter LGT) are responsible for ex situ conservation. Several zoos also maintain giant pandas for education, exhibition, and breeding, such as the Beijing Zoo (hereafter BJ) (State Forestry Administration of China 2006; Wei et al. 2006, 2012).

Before the 1990s, low conception rates, sexual incompatibility, and high neonatal mortality impeded efforts to breed giant pandas. During that time, only 30% of captive giant pandas reproduced successfully with neonatal mortality of more than 60% (Peng et al. 2001; Zhang et al. 2006). To overcome the behavioral incompatibility between sexes, artificial insemination with fresh/frozen-thawed sperm was successfully developed and is now routine at Chinese breeding centers (Wei et al. 2012). Another important achievement in captive breeding was increasing cub survival rate. Of the 60% historic neonatal mortality, a large proportion was "natural" discard of a twin by the panda mother. The successful development and application of partial/complete hand rearing of cubs, commercial milk formulae, and "twin swapping" techniques have resulted in a steady rise in neonatal survival to 71% from 1990 to 2002 (Zhang et al. 2006). By 1997, the number of captive-born giant pandas outnumbered wild-born animals in the ex situ population (Zhang et al. 2006) and by the end of 2012, 341 animals were in captivity (Xie and Gipps 2012). This success means that plans are now underway to release captive-born animals into the wild (Wei et al. 2012).

A successful captive breeding program requires not only a large population, but also ample genetic variation to act as a genetic resource bank for wild populations. There is much concern and uncertainty regarding the genetic status and inbreeding levels of captive giant pandas. First, it is often thought that the captive population is small, distributed across many facilities (67), and suffering from severe inbreeding (Zhang and Wang 2003; Ballou et al. 2006; Zhang and Wei 2006; Xie and Gipps 2012). Second, although the movement of animals between different captive populations is recorded (Xie and Gipps 2012) and initial genetic work has been done using a small sample size (Shen et al. 2009), more detailed genetic assessments of gene flow and genetic structure across the whole captive population are lacking. Third, as the population of captive animals grows, whether to introduce new wild individuals or their genetic material into the captive population is a hot topic amongst conservationists, captive breeders, and managers (Wildt et al. 2006; Shen et al. 2009). Given the current state of play of giant panda captive breeding and that captive-born animals will soon be systematically released into the wild, it is crucial to assess the genetic status and inbreeding levels of the entire captive population. Here, we sampled a large number of captive individuals (240 giant pandas or 64% of animals in captivity) and combined mitochondrial DNA (mtDNA) control region (CR) sequences and 19 microsatellite markers to assess genetic variation and inbreeding levels. Our goals were to clarify the three above-mentioned concerns using qualitative and quantitative genetic analyses, identify scientific strategies to aid ongoing ex situ giant panda conservation programs, and inform programs planning to release captive-born animals into the wild.

Results

Genetic Diversity

We successfully obtained 655 bp of mtDNA CR sequences from 220 captive giant pandas and genotyped all 240 animals across 19 microsatellite loci. We combined the mtDNA data with 245 mtDNA CR sequences previously obtained from wild giant pandas, and identified 26 polymorphic sites and 41 unique haplotypes in total, including 19 haplotypes for the captive population (fig. 1; supplementary table S1, Supplementary Material online). Seventeen of the 19 haplotypes are shared between captive and modern wild samples, and another two (GH04 and GH34) are identified from historical skin samples in Zhu et al. (2013). Most of the high-frequency CR haplotypes such as GH03 (16%), GH10 (17%), and GH35 (7%) were shared between the captive and wild populations (fig. 1). Haplotype diversity (h) ranged from 0.7 to 0.889 and from 0.532 to 0.9 for the captive and wild populations, respectively, and nucleotide diversity (π) ranged from 0.0047 to 0.0062 and from 0.0018 to 0.0067 for the captive and wild populations, respectively. Based on analysis of microsatellite data, the mean number of alleles (MNA) per locus ranged from 4.1 to 5.8 and from 3.5 to 7.63 for the captive and wild populations, respectively. Expected heterozygosity (H_E) is 0.578 to 0.666 for the captive population and 0.486 to 0.803 for wild populations (table 1). When the genetic diversity of captive populations was compared with wild populations, mean differences in genetic variation for CR (i.e., number of polymorphic sites [N_V] and π) and microsatellite data (i.e., MNA, observed heterozygosity [H_O] and H_E) were not significant (ANOVA, $P > 0.05$). These comparative analyses imply that a relatively high-level of genetic diversity is preserved in captive populations and is comparable to that of wild populations.

Estimated Individual Inbreeding and Pairwise Relatedness

Based on 19 microsatellite loci, the individual inbreeding and pairwise relatedness values were estimated. We found that 78.3% (188) of the 240 sampled individuals, 65.2% (30) of the 46 wild born and 81.4% (158) of the 194 captive born have an estimated inbreeding coefficient of $f < 0.125$ (fig. 2a), and the average is 0.169, 0.073, 0.066, 0.024, and 0.074 for LGT, CD, WL, BJ, and the whole population, respectively. However, within the LGT population, 61.9% of the individuals (13 out of 21) have an estimated inbreeding coefficient of $f \geq 0.125$, and 23.8% have $f \geq 0.25$. Genetic relatedness analysis showed that 77.0% of pairwise individuals among the whole population, 81.4% among the 46 wild-born individuals, and 74.9% among the 194 captive-born individuals have an estimated relatedness value of $r < 0.125$ (fig. 2b); the average is 0.139, 0.025, 0.042, 0.008, and −0.003 for LGT, CD, WL, BJ, and the whole population, respectively. Among the four captive populations studied here, LGT has the highest estimated inbreeding coefficient and relatedness value.

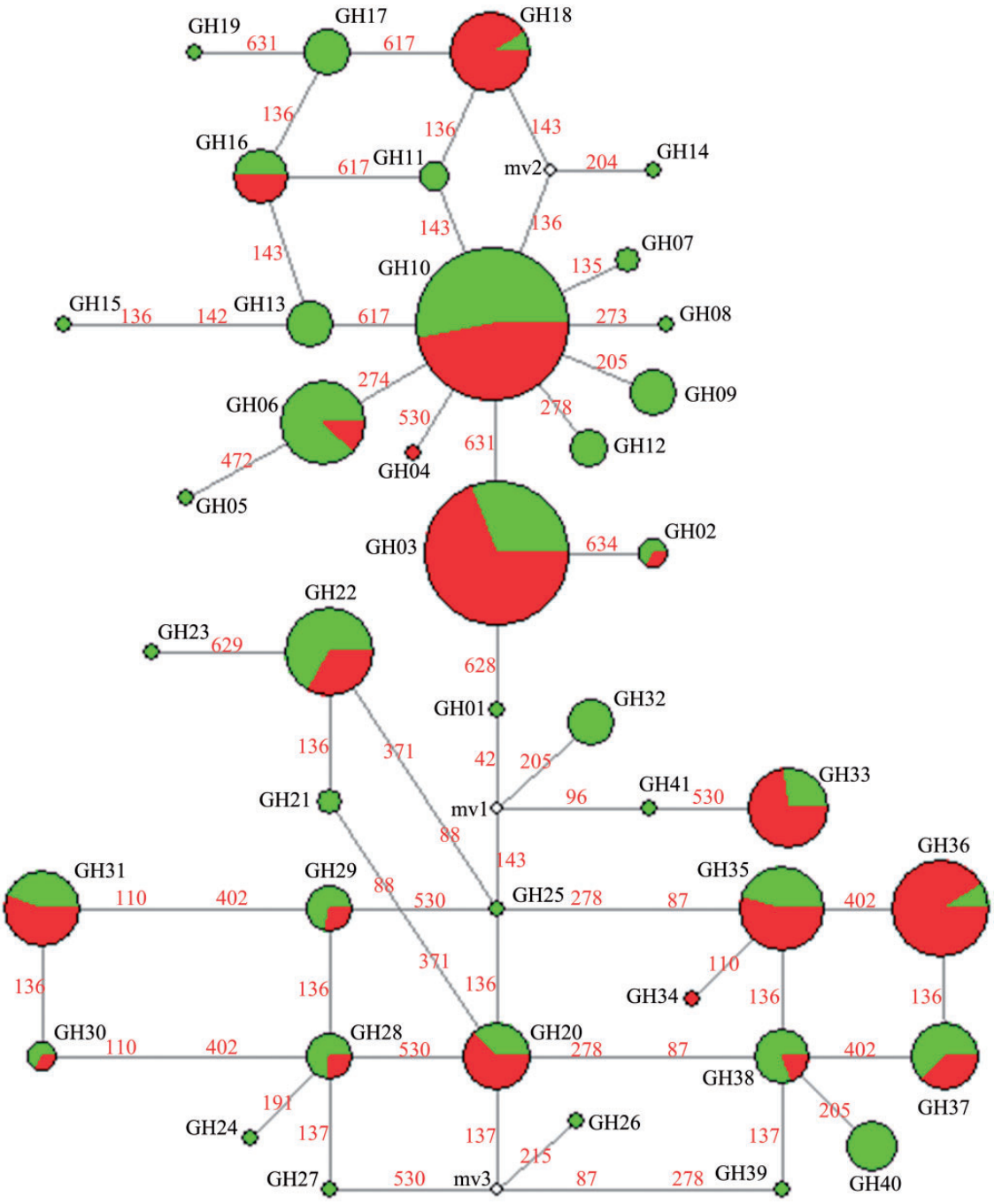

FIG. 1. The distribution of mitochondrial haplotypes of giant pandas using the median-joining method implemented in Network 4.6.1.2. Captive: red; Wild: green.

Genetic Structure

Bayesian clustering revealed strong genetic structuring ($K = 2$) among captive populations. Individuals from LGT and CD predominantly formed one genetic cluster, being significantly differentiated from WL and BJ, which formed the other genetic cluster (fig. 3). For mtDNA and microsatellite data, the fixation index (F_{ST}) showed similar results: significant F_{ST} values were detected between populations, except for WL versus BJ based on mtDNA (table 2). The analysis of molecular variance (AMOVA) for mtDNA strongly supported the results produced by F_{ST} analysis based on mtDNA, detecting three genetic groupings (LGT, CD, and WL–BJ) among populations ($F_{CT} = 0.198$, $P < 0.001$; table 3). These results indicate that significant genetic differentiation has occurred among giant panda breeding centers.

Discussion

Relatively high levels of genetic diversity have been preserved in captive giant panda populations, are comparable to that in wild populations, and much better than for other high-profile ex situ breeding programs such as for the black-footed ferret (Wisely et al. 2002) and California condor (Ralls and Ballou

Table 1. Estimates of Genetic Diversity of Giant Pandas in Captivity and in the Wild.

Population	*N* (CR/MS)	N_H	N_V	*h*	π	MNA	H_O	H_E	Reference
Captive									
LGT	17/21	8	11	0.816	0.0050	4.1	0.516	0.578	This study
CD	63/67	8	14	0.700	0.0047	5.2	0.658	0.645	This study
WL	130/138	14	13	0.850	0.0059	5.8	0.615	0.619	This study
BJ	10/14	6	8	0.889	0.0062	4.3	0.688	0.648	This study
CAP	220/240	19	16	0.880	0.0061	6.3	0.623	0.641	This study
Chengdu	–/49	–	–	–	–	5	0.671	0.634	Shen et al. 2009
Wolong	–/34	–	–	–	–	5.55	0.672	0.666	Shen et al. 2009
Wild									
QIN	37/32	11	13	0.884	0.0062	3.5	0.525	0.486	Zhu et al. 2013; Zhang et al. 2007
MIN	69/29	16	15	0.900	0.0067	4.8	0.561	0.559	Zhu et al. 2013; Zhang et al. 2007
QIO	46/40	11	12	0.851	0.0053	5.3	0.595	0.610	Zhu et al. 2013; Zhang et al. 2007
DXL	21/21	5	5	0.748	0.0018	4.7	0.660	0.634	Zhu et al. 2013; Zhu et al. 2011
XXL	32/32	5	10	0.532	0.0020	4.6	0.704	0.656	Zhu et al. 2013; Zhu et al. 2011
LIS	40/52	10	12	0.763	0.0052	4	0.683	0.592	Zhu et al. 2013; Hu et al. 2010
WIL	245/–	39	26	0.937	0.0068	–	–	–	Zhu et al. 2013
Wanglang	–/74	–	–	–	–	5.4	0.625	0.609	Zhan et al. 2006
Wanglang	–/31	–	–	–	–	5.64	0.520	0.694	Shen et al. 2009
Baoxing	–/25	–	–	–	–	7.63	0.483	0.803	Shen et al. 2009

NOTE.–MS, microsatellite; N_H, number of haplotypes; N_V, number of polymorphic sites; *h*, haplotype diversity; π, nucleotide diversity; H_O, observed heterozygosity; H_E, expected heterozygosity; LGT, Shaanxi (Louguantai) Rescue and Breeding Center for Rare Wildlife; CD, Chengdu Research Base of Giant Panda Breeding; WL, China Research and Conservation Center for the Giant Panda at Wolong; BJ, Beijing Zoo; CAP, all the captive giant pandas in this study; QIN, Qinling Mountains; MIN, Minshan Mountains; QIO, Qionglai Mountains; DXL, Daxiangling Mountains; XXL, Xiaoxiangling Mountains; LIS, Liangshan Mountains; WIL, all CR data from modern wild samples used in this study; Wanglang, a nature reserve located in MIN Mountains; Baoxing, a nature reserve located in Qionglai Mountains.

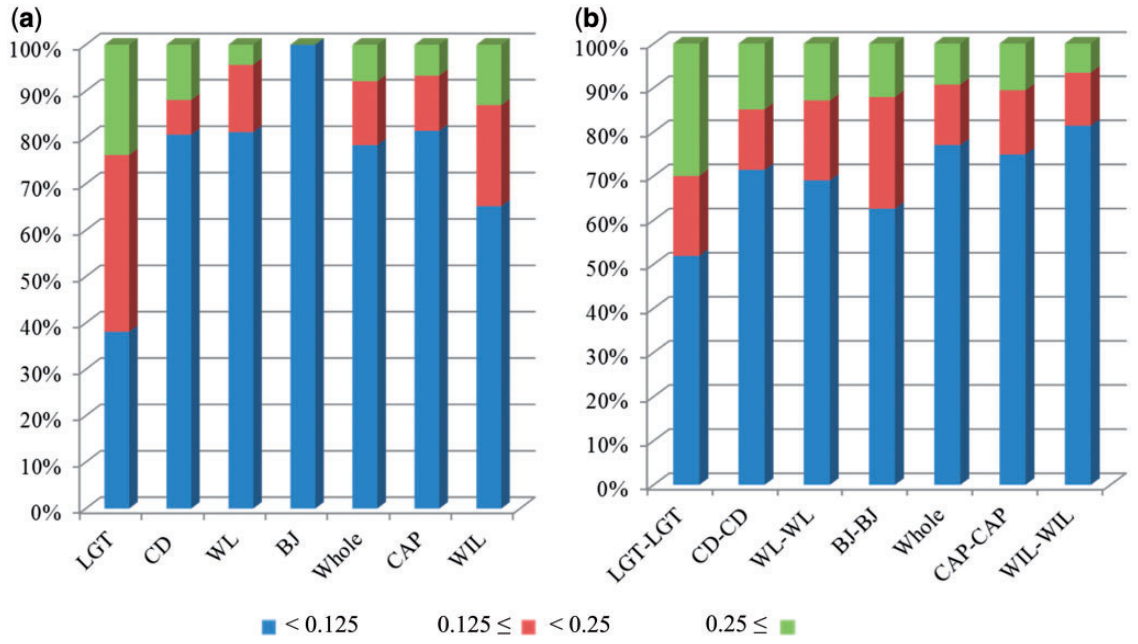

FIG. 2. The distributions of (*a*) estimated individual inbreeding coefficients, (*b*) estimated pairwise relatedness values. LGT, Shaanxi (Louguantai) Rescue and Breeding Center for Rare Wildlife; CD, Chengdu Research Base of Giant Panda Breeding; WL, China Research and Conservation Center for the Giant Panda at Wolong; BJ, Beijing Zoo; Whole, all of the 240 giant pandas; CAP, captive-born individuals among the 240 giant pandas; WIL, wild-born individuals among the 240 giant pandas.

2004). This result means it is unnecessary to add further genetic material from wild animals into captive populations on the whole. However, this finding is inconsistent with a previous study (Shen et al. 2009) that detected low levels of genetic diversity in ex situ populations using 11 microsatellite loci across 83 captive giant pandas and proposed the addition of genetic material from wild giant pandas. This discrepancy may be because of differences in sample size and sampling range. Shen et al. (2009) sampled 83 captive individuals from the CD and WL captive populations only and compared the

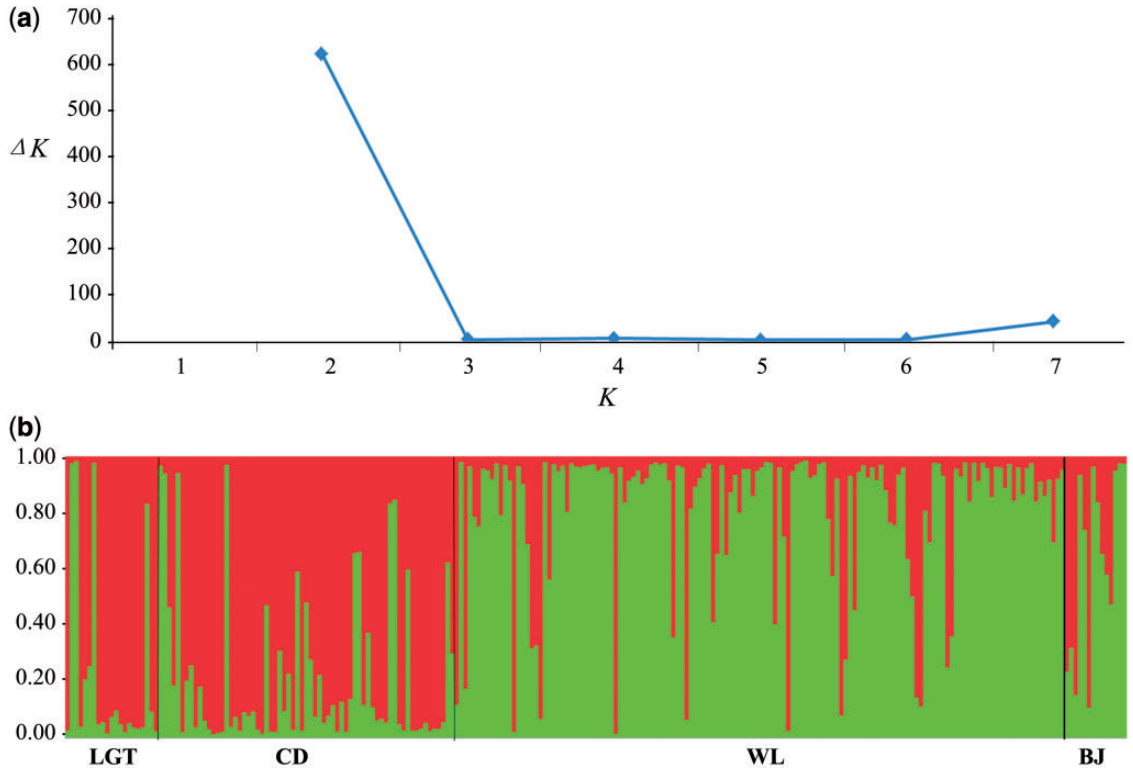

FIG. 3. Bayesian STRUCTURE clustering results based on microsatellite genotypes among four captive giant panda populations. (*a*) ΔK values as a function of K based on ten runs indicating the most likely number of two genetic clusters, (*b*) STRUCTURE output of two genetic clusters identified ($K = 2$), represented by the colors red and green. The proportions of ancestry assigned to different clusters were plotted by individuals. LGT, Shaanxi (Louguantai) Rescue and Breeding Center for Rare Wildlife; CD, Chengdu Research Base of Giant Panda Breeding; WL, China Research and Conservation Center for the Giant Panda at Wolong; BJ, Beijing Zoo.

Table 2. Pairwise F_{ST} Estimates.

	LGT	CD	WL	BJ
LGT		0.047***	0.057***	0.054***
CD	0.322***		0.044***	0.031***
WL	0.262***	0.144***		0.017*
BJ	0.237*	0.177*	−0.014	

NOTE.—Pairwise F_{ST} values based on microsatellite (above diagonal) and mtDNA CR data (below diagonal). Significance was indicated after Bonferroni correction: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 3. Analysis of molecular variance (AMOVA) for groupings for captive giant panda populations based on mtDNA control region (CR) sequence.

	Grouping	F_{CT}	*P* value
Group 1	LGT–CD, WL–BJ	−0.041	0.618
Group 2	LGT–CD, WL, BJ	−0.203	0.993
Group 3	LGT, CD–WL–BJ	0.166	0.001
Group 4	LGT, CD–WL, BJ	0.073	0.102
Group 5	LGT, CD, WL–BJ	0.198	0.000

genetic diversity to that of only two nature reserve populations. In contrast, our study sampled the four largest captive populations and compared genetic diversity with all six wild giant panda populations.

As reflected by the estimates, inbreeding is managed effectively at a low level across the whole captive population. Most (78.3%) of the individuals have an estimated inbreeding coefficient of < 0.125 and most (77.0%) pairwise individuals have an estimated relatedness value of < 0.125. This is likely attributable to 1) the current breeding pairing plan recommended annually by the Chinese Committee of Breeding Techniques for Giant Pandas under the direction of the Chinese Association of Zoological Gardens based on pedigree information, 2) and the significant number of wild founders in the ex situ population. For instance, of the 859 animals recorded in the studbook so far (Xie and Gipps 2012), 311 (36.2%) have been wild caught, whereas of our 240 sampled individuals, 46 (19.17%) were wild-born.

Although it is exciting that a genetically healthy population of giant pandas has been successfully established in captivity on the whole, our analyses did reveal issues at the breeding center level that warrant exploration. The LGT center was originally founded to rescue giant pandas facing nutrition or health problems in the Qinling Mountains. However, this population now has a higher chance of inbreeding than other captive populations, as indicated by estimated inbreeding coefficients and relatedness values (fig. 2). This population remains very small (only 19 living individuals according to the 2012 studbook) and requires urgent attention such as scientific breeding pairing or genetic input from rescued wild giant pandas.

Our genetic clustering analysis showed significant genetic structuring among captive populations. Specifically, LGT–CD

was genetically clustered, as was WL–BJ. This genetic structure is basically consistent with the history of individual breeding exchange between captive panda populations (Xie and Gipps 2012). However, mtDNA-based AMOVA analysis classified LGT and CD as two distinct groups. This difference could be explained by different genetic patterns of mtDNA (maternally inherited) and nuclear microsatellite marker (biparentally inherited) under the context of individual exchange. For example, we examined the geographic sources of 21 LGT individuals and found that 12 individuals originated from the wild Qinling Mountains population, four individuals originated from the non-Qinling wild population, and five individuals originated from hybridization between Qinling and non-Qinling animals. As a result, the mtDNA haplotypes of 15 individuals originated from the Qinling wild population and genetic distance-based AMOVA analysis identified LGT as a distinct group. According to biparentally-inherited microsatellite analysis, nine individuals transmitted genetic information from the non-Qinling wild population, which would mix population-specific microsatellite alleles, significantly change allele frequencies and result in the clustering of LGT and CD. Although gene flow between different captive centers is always encouraged by captive breeding specialists in order to avoid inbreeding and genetic fragmentation (Ballou et al. 2006), the exchange between LGT and CD is problematic because of the unique status of the LGT population representing the wild Qinling population. The Qinling population diverged from non-Qinling populations (Minshan [MIN], Qionglai-Daxiangling-Xiaoxiangling-Liangshan [QXL]) approximately 0.3 Ma and local adaptation has occurred in the Qinling and non-Qinling populations (Zhao et al. 2013). This adaptation may be illustrated by differences in morphology (Wan et al. 2005). The CD population was established by founders mainly from the MIN and Qionglai populations (non-Qinling populations), and thus, the exchange between LGT and CD is essentially an exchange between Qinling and non-Qinling wild populations. This exchange may disrupt patterns of local adaptation established over hundreds of thousands of years, and thus would result in an unpredictable impact on wild populations if captive individuals were reintroduced into their original environments (Wei et al. 2012; Zhao et al. 2013). Frankham (2010) suggested that interbreeding between differentiated populations should be allowed in order to rescue endangered populations, although outbreeding depression remains to be assessed. This view holds true in some cases, however, in the case of the LGT captive population, conservation strategies such as scientific breeding pairing and the addition of genetic material from wild individuals rescued because of nutrition or health problems should be given the highest priority to effectively avoid inbreeding and maintain genetic diversity. We suggest that this LGT population with a pure Qinling source be managed independently to genetically resemble its wild counterparts under a long-term view of reintroduction, and that gene flow between LGT and CD should be avoided because of the genetic distinctiveness of the Qinling population (Lu et al. 2001; Zhang et al. 2007; Zhao et al. 2013). In contrast, CD and WL populations have little gene flow, albeit both of their wild founders originated from the non-Qinling populations (Xie and Gipps 2012). Within the non-Qinling populations, the divergence time of the MIN and QXL mountain populations was relatively recent (Zhao et al. 2013). Therefore, the exchange between CD and WL, the two largest breeding centers, should be encouraged to better retain genetic diversity.

Although the reintroduction of captive individuals has only been undertaken in the small and isolated Xiaoxiangling population (Zhu et al. 2010; Qi et al. 2012; Wei et al. 2012), there are plans to expand this practice across the giant panda range. The proper selection of captive individuals is critical work that could affect the success of reintroductions. However, to our knowledge, the already implemented reintroduction programs do not consider genetic compatibility between wild target populations and reintroduced animals. Our large-scale genetic survey provides the most comprehensive genetic database of captive populations thus far and includes information regarding genetic variation, structure, and genetic relationships. Combined with previous findings on wild populations, this database will clarify the genetic contribution of reintroduced animals to wild populations.

Traditionally, the estimation of inbreeding coefficients and relatedness values in the studbook are based on the assumption that founders are unrelated and noninbred (Ballou and Lacy 1995; Witzenberger and Hochkirch 2011). This assumption has facilitated breeding pairing management based on pedigree data to reduce inbreeding effectively in captivity (Lacy et al. 1995; Ballou et al. 2006; Xie and Gipps 2012). However, our results show that the assumption is far from the truth. For example, 34.8% of the 46 wild-born individuals were estimated to have moderate to high levels of inbreeding ($f \geq 0.125$) (fig. 2*a*), and 18.6% of pairwise individuals were estimated to have a close kinship ($r \geq 0.125$) (fig. 2*b*). These findings indicate that many of the founders are genetically related, and pairing based on this assumption would result in inbred offspring. Therefore, combining our genetic data with pedigree data could better guide the pairing of captive giant pandas and further reduce inbreeding in the future.

In conclusion, this study is the most comprehensive genetic survey of the captive giant panda population to date. Using a large-scale genetic database, we found that the captive population is genetically healthy and self-sustaining on the whole, as indicated by ample genetic variation, low levels of inbreeding, and a large captive population size. This is an exciting result and the culmination of recent decades of captive breeding. Although the big picture is positive, our genetic survey reveals some important but previously cryptic problems in captive management, such as the poor genetic status of the LGT population and its exchange with CD, the scant exchange between CD and WL, traditional breeding pairing, and the neglect of genetic backgrounds when selecting captive individuals for reintroduction into the wild. We hope that the issues and suggestions identified in this study enhance the captive giant panda population as this high-profile program transitions to the release of captive-born animals into the wild.

Materials and Methods

Sample Collection and Data

We collected blood samples from 240 captive giant pandas in four main captive institutions (each considered a population): LGT (n = 21), CD (n = 67), WL (n = 138), and BJ (n = 14). From this sample set, 217 animals were alive at the end of 2012, representing approximately 64% of the 341 living individuals listed in the 2012 International Giant Panda Studbook (Xie and Gipps 2012). Samples were obtained from giant pandas more than 2 years old during routine medical examinations with permission from the State Forestry Administration of China. All 240 individuals were microsatellite-genotyped, and 655 bp of mtDNA CR fragments were analyzed successfully for 220 animals. In addition, we used genetic diversity information derived from microsatellite genotypes of 83 captive and 475 wild giant pandas (see details in table 1), and 245 mtDNA CR sequences from modern samples of wild giant pandas of known geographical origin from a previous study (Zhu et al. 2013; supplementary table S1, Supplementary Material online).

DNA Extraction and Amplification

Genomic DNA was extracted with a DNeasy Blood/Tissue Kit (QIAGEN), following the manufacturer's instructions. The 5′-end of mtDNA CR (655 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′) and BEDH (5′ -GGG TGA TCT ATA GTG TTA TGT CC- 3′) (Zhang et al. 2007). PCR fragments were sequenced in an ABI 3730xl automated DNA analyzer.

Nineteen giant panda-specific microsatellite loci, Ame-μ5, Ame-μ10, Ame-μ13, Ame-μ14, Ame-μ15, Ame-μ22, Ame-μ26 (Lu et al. 2001), Ame-μ11, Ame-μ24, *Aime*-1, *Aime*-10, *Aime*-13, AY79, AY87, AY95, AY99 (Wu et al. 2009), AY161198, AY161213 (Shen et al. 2005), and g^{901} (Zhang et al. 1995) were selected for this study after initial assessment based on PCR efficiency, polymorphism and scoring performance. PCR was performed in a 10 μl volume containing 1 μl DNA, 5 μl Premix ExTaq (TaKaRa), 0.2 μM of forward primer end-labelled with a fluorescent dye (FAM, HEX, or TAMRA), and 0.2 μM reverse primer. PCR amplification was carried out with an initial step of 94 °C for 5 min, followed by a touchdown PCR (a total 21 cycles of 95 °C/15 s, T_m/30 s, 72 °C/45 s), and a final step of 72 °C for 15 min. T_m was decreased by 0.5 °C/cycle starting from 60 °C to a final temperature of 50 °C, which was used for the next 25 cycles. PCR products were genotyped in an ABI 3730xl automated DNA analyzer and scored using GeneMarker 2.2 (SoftGenetics). To obtain reliable genotypes, amplification was repeated minimally three times. We scored samples as heterozygous at a locus if both alleles appeared at least twice among replicates and as homozygous if all the replicates showed identical homozygous profiles. If neither of those cases applied, we repeated additional amplification until fitting the criteria. The final microsatellite genotypes were assessed for the presence of null alleles, large allele dropout, and stuttering using MicroChecker v2.23 (Van Oosterhout et al. 2004). No evidence of null alleles or associated genotyping errors was found for any locus.

MtDNA Data Analysis

Individual mtDNA CR sequences were edited by DNASTAR 7.1 (Burland 2000) and consensus haplotypes were aligned using Mega 5.2 (Tamura et al. 2011), and then rechecked by eye. Nucleotide diversity (π, Nei 1987), haplotype diversity (h, Nei 1987), and number of variable sites (N_V) were estimated for the whole data set and for each population, respectively, using ARLEQUIN 3.5 (Excoffier et al. 2005) and DNASP 5.0 (Librado and Rozas 2009). Genetic differentiation (F_{ST}) and molecular variance analysis (AMOVA; Excoffier et al. 1992) were performed to test for differentiation between different captive populations using ARLEQUIN 3.5. NETWORK (Bandelt et al. 1999) was used to reconstruct phylogenetic relationships among haplotypes with a median-joining network method.

Microsatellite Data Analysis

MNA per locus, H_O and H_E were calculated using ARLEQUIN 3.5. The linkage disequilibrium (LD) across all pairs of loci was tested using FSTAT 2.9.3.2 (Goudet 2001), and no LD was found for any pairs of loci in any population after Bonferroni sequential correction (Rice 1989).

A Bayesian clustering method implemented in STRUCTURE 2.3.1 (Pritchard et al. 2000) was used to determine the most likely number of genetic clusters regardless of where they were collected. The admixture model was chosen, allele frequencies were assumed correlated and analyses were conducted with a burn-in of 100,000 and followed by 1,000,000 MCMC repetitions. Ten independent runs were carried out for each cluster set (K) from 1 to 8. The most likely K value was determined by evaluating the log likelihood [ln P (X/K)] of the posterior probability of the data for each value of K (Pritchard et al. 2000). Further, the ΔK statistic, the second-order rate of change in the log probability of the data between successive values of K, was also estimated and used to determine the most likely number of genetic clusters (Evanno et al. 2005). Genetic differentiation (F_{ST}) between populations was estimated using ARLEQUIN 3.5, and statistical significance of F_{ST} values was tested with 10,000 permutations.

The QuellerGt moment estimator (Queller and Goodnight 1989) and triadic maximum likelihood (TrioML) estimator (Wang 2007) implemented in Coancestry 1.0 (Wang 2011) were used to estimate pairwise relatedness value (r) between two individuals and inbreeding coefficient (f) for each individual, respectively. A negative estimated relatedness value suggests distant kinship and positive value suggests that an individual pair is related. Individual inbreeding coefficient reflects the extent to which their parents are genetically related. Estimates using other models implemented in Coancestry 1.0 were compatible with those of QuellerGt and TrioML (supplementary table S2, Supplementary Material online).

Following Marshall et al. (2002), $f < 0.125$ is defined as low inbreeding, $0.25 > f \geq 0.125$ as moderate, and $f \geq 0.25$ as high.

Supplementary Material

Supplementary tables S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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