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INVITED REVIEWS AND META-ANALYSES

Black and white and read all over: the past, present and future of giant panda genetics

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Abstract

Few species attract much more attention from the public and scientists than the giant panda (*Ailuropoda melanoleuca*), a popular, enigmatic but highly endangered species. The application of molecular genetics to its biology and conservation has facilitated surprising insights into the biology of giant pandas as well as the effectiveness of conservation efforts during the past decades. Here, we review the history of genetic advances in this species, from phylogeny, demographical history, genetic variation, population structure, noninvasive population census and adaptive evolution to reveal to what extent the current status of the giant panda is a reflection of its evolutionary legacy, as opposed to the influence of anthropogenic factors that have negatively impacted this species. In addition, we summarize the conservation implications of these genetic findings applied for the management of this high-profile species. Finally, on the basis of these advances and predictable future changes in genetic technology, we discuss future research directions that seem promising for giant panda biology and conservation.

Keywords: adaptation, conservation genetics, evolution, giant panda, molecular ecology

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Introduction

On seeing this review, it may occur to the reader to ask: why is a review on the past, present and future of giant panda (*Ailuropoda melanoleuca*) genetics needed? The giant panda is certainly not a model organism: indeed, it is the opposite of a species whose biology would make it appropriate for concerted genetic studies. For instance, unlike other carnivores, the giant panda is not widespread and does not possess a large variety of locally adapted forms that could yield insight into ongoing evolutionary processes. The giant panda is far from being a fecund species (Zhang & Wei 2006), and its reproductive behaviours are rarely observed. There is not yet even a very large body of literature on the genetics of giant pandas. These factors make the choice of this particular species for a review in *Molecular Ecology* an apparently strange one at first.

On the other hand, the giant panda is a species whose allure is difficult to ignore. The species enjoys an iconic status worldwide. Its unrivalled political status and conservation history over the last 50 years are well documented (Nicholls 2010). It is also, importantly, a controversial species, with a widely held opinion that condemns it as being maladapted and poorly suited to its present environment and 'incompetent in almost every function crucial to its survival' (Catton 1990). Its status as a highly endangered species and its candidacy for imminent extinction are often seen as a self-fulfilling prophecy.

But, there are other reasons why an understanding of the evolutionary and population genetics of the giant panda is inherently interesting. First, the giant panda is a highly distinct taxon: the most basal species in the phylogeny of living bears (O'Brien *et al.* 1984, 1985), whose closest relative (i.e. the spectacled bear,

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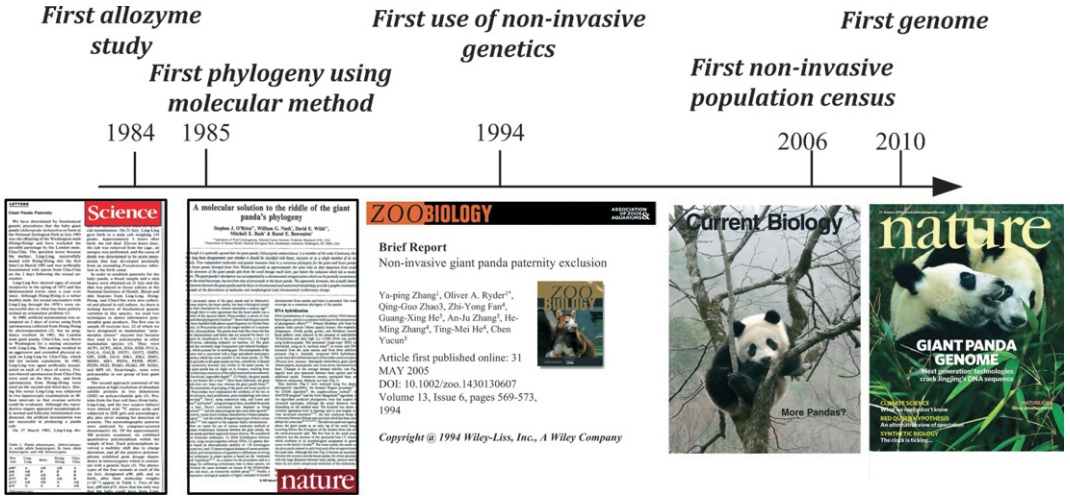
Tremarctos ornatus) is not very closely related at all and is restricted to mountain range on another continent (South America). Palaeontological studies suggest that while *Ailuropoda* was once found as far north as the mountains surrounding Beijing, and as far south as Myanmar, the genus has only ever had two or three species, and *A. melanoleuca* has been monotypic for 3–4 millions years (Wang 1974). As the sole remaining representative of a distinct evolutionary lineage among the carnivores, with an almost exclusively bamboo-based diet, unique morphological and physiological adaptations and possessing its characteristic black and white pelage, the giant panda provides compelling examples of adaptive differentiation from other members of its evolutionary lineage. In addition, the conservation of giant panda's landscape and critical habitat provides a protective 'umbrella' for many other distinct species in what is one of the world's top 25 biodiversity hotspots. The presence of the giant panda in a region under great pressure from anthropogenic development (for the last several thousand years) has provided protection against habitat loss and fragmentation not afforded elsewhere in mainland East Asia.

Even when not being considered in an ecological context, the giant panda's status as a species worthy of the attention of geneticists remains clear. Its role in the development of *ex situ* conservation during the 20th and 21st century has been very important. This is largely due to its immense popularity as a species maintained in zoos and its critical role in focusing the development of assisted reproductive technologies and captive breeding. Now, captive breeding of giant pandas is so successful that assisted reproduction is routine in China and has occurred elsewhere in the world, providing a potential fund of individuals for reintroduction into the wild. Management of captive-bred species and especially the maintenance of genetic diversity through pedigree management are issues that have been increasingly addressed against the backdrop of managing high-profile species for potential reintroduction.

Given the factors described above, the aim of our article is to highlight aspects of the biology of the giant panda that have been uniquely clarified by genetic data. It could be argued that the conservation of this species has been influenced by genetics in a way unparalleled in almost any other endangered species. By clarifying the giant panda's evolutionary position, understanding its genetic diversity across its geographical range, reconstructing its demographical history, applying non-invasive genetics in population census, illuminating key natural history knowledge gaps such as sex-specific dispersal, habitat selection and chemical communication, and finally to the recently published genome and metagenome, the relevance of genetic data on the conservation and management of this species has been very clear during the past 20 years (Box 1). Perhaps, the one question that evolutionary and population genetics is uniquely placed to ask is to what extent the giant panda's current status is a reflection of its evolutionary legacy, as opposed to the influence of anthropogenic factors that have negatively impacted many other endangered species in a similar manner. It is also our aim in this article to shed light on whether this question has been fully addressed and what is needed in the future to finally conclude it.

Box 1
Timeline of molecular genetic research in giant pandas (key milestones)



Evolution and phylogenetic distinctiveness

Historically, giant pandas ranged through southern, middle and north-west China into northern Myanmar, northern Vietnam, Laos and Thailand, but currently are confined to six mountain ranges on the eastern edge of the Tibetan Plateau in China: Qinling, Minshan, Qionglai, Liangshan, Daxiangling and Xiaoxiangling (Fig. 1; Hu 2001). Based on a skin specimen collected by the French missionary Père Armand David in 1869 in Baoxing, Sichuan Province, Milne Edwards identified this as a distinct species, the giant panda (*Ailuropoda melanoleuca*). Since that time, its taxonomy and systematics have been continuously debated.

Early evolutionary studies focused on morphology and anatomy. Three hypotheses prevailed (i) that it belongs to Procyonidae (e.g. Mivart 1885; Lankester 1901), (ii) that it belongs to Ursidae (e.g. Davis 1964; Sarich 1973) and (iii) that it belongs to its own family Ailuropodidae (e.g. Pocock 1928; Zhu 1974). Since the 1980s, molecular genetics has offered a potential solution to this issue. During the 1980s and 1990s, researchers unanimously rejected the first hypothesis, agreeing that the giant panda is not a relative of the raccoon (*Procyon lotor*), instead supporting the second hypothesis that it belongs to the bear lineage. Allozyme, DNA–DNA hybridization, immunology and karyotyping data all indicated that the panda and other modern bears share a common ancestor (Fig. 2a; O'Brien *et al.* 1984, 1985). Furthermore, using mitochondrial cytochrome *b* and $tRNA^{Thr}$ and $tRNA^{Pro}$ data for Ursids, phylogenetic analyses have indicated that the giant panda and the spectacled bear are basal taxa within the Ursid radiation (Talbot & Shields 1996). In the 1990s, some studies advocated the classification of a family Ailuropodidae within the Arctoidea, based on genetic divergence data using mitochondrial (mt) DNA restriction fragment length polymorphisms (Zhang & Shi 1991) and mtDNA sequencing (Zhang & Ryder 1993). However, these studies failed to gain a consensus phylogenetic conclusion for the Ursidae (Zhang & Ryder 1994; Flynn & Nedbal 1998).

Since the 2000s, researchers have tried to resolve the debate by applying different markers from the nuclear genome. The consensus has been reinforced that the panda is indeed a bear (e.g. based on one exon and one intron marker, Yu *et al.* 2004; 14 nuclear genes including X-, Y-linked and autosomal genes, Pagès *et al.* 2008; 14 nuclear genes, Eizirik *et al.* 2010), including an important recent study using a data set comprising >22 kb of nuclear intron loci in 16 caniformian species (Fig. 2b, Yu *et al.* 2011). However, these studies have not been able to clarify the branching order among the Ursidae.

Further, some studies have suggested that the Qinling population should be considered as a separate subspecies (*A. m. qinlingensis*) with the remaining populations being classified as *A. m. melanoleuca*, based on significant differences in DNA fingerprinting profiles and morphological characters (Wan *et al.* 2003, 2005). Zhang *et al.* (2007) also found significant genetic differentiation between Qinling and four other extant populations based on mtDNA D-loop sequences and 10 microsatellite loci.

Today, with more and more genome sequences becoming available, genome-level phylogenetic analysis is probably to resolve many debates of this nature (e.g.

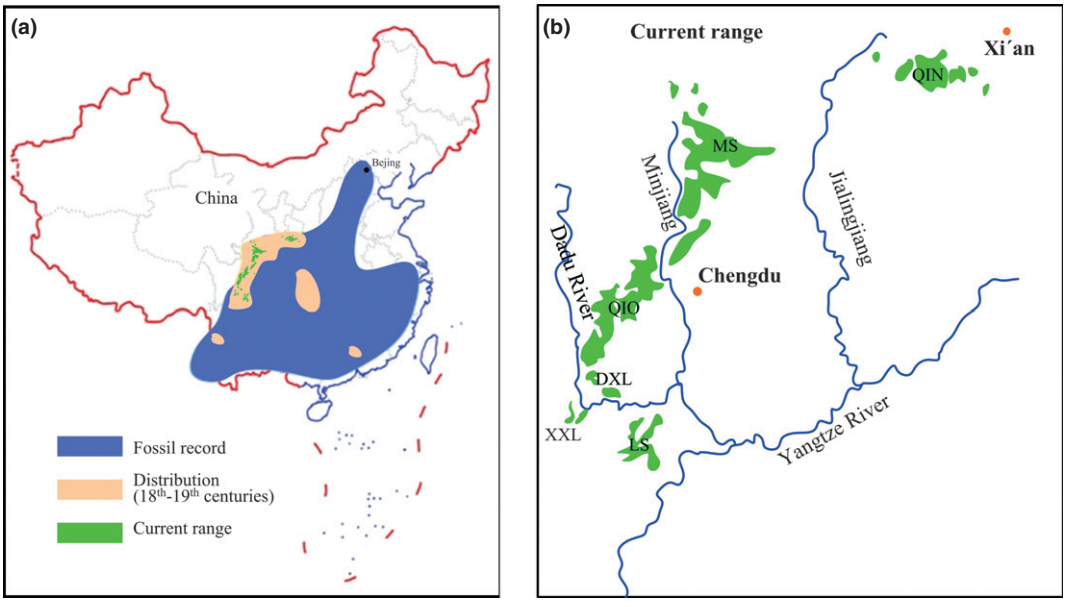


Fig. 1 The historical (a) and current range (b) of giant panda (redrawn from Loucks *et al.* 2001; State Forestry Administration 2006; Zhang *et al.* 2007). QIN, Qinling Mountains; MS, Minshan Mountains; QIO, Qionglai Mountains; LS, Liangshan Mountains; DXL, Daxiangling Mountains; XXL, Xiaoxiangling Mountains.

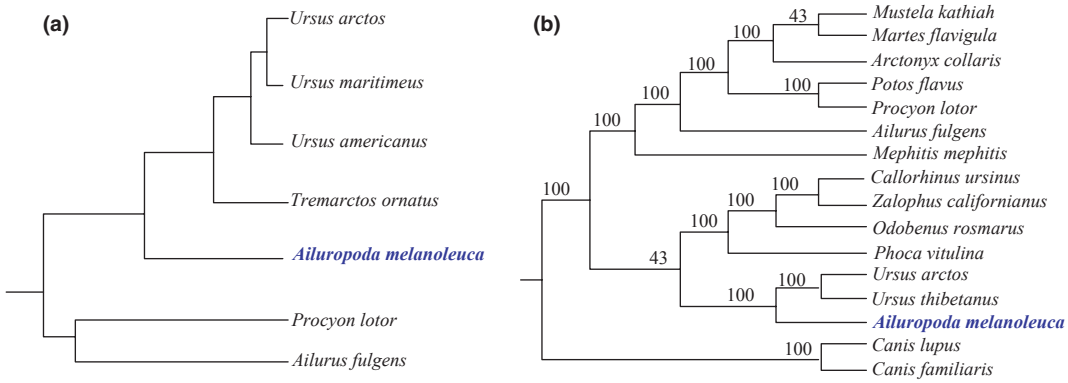


Fig. 2 Phylogenetic position of the giant panda. (a) Phylogenetic tree based on immunological, DNA–DNA hybridization and isozyme evidence (redrawn from O'Brien *et al.* 1985); (b) maximum-likelihood phylogenetic tree based on combined nuclear intron analyses (redrawn from Yu *et al.* 2011).

Smith *et al.* 2011) and the recently published panda genome will transform genetic studies for this species (Li *et al.* 2010), providing an essential tool for a detailed understanding of the biology of this organism and its phylogenetic position. Thus, phylogenomic analysis (combining with genome data for other Ursidae species) is expected to resolve the most pressing question in the near future: the detailed phylogenetic relationships within the Ursidae.

Genetic diversity

Populations with higher genetic diversity are often thought to have greater options to adapt to environmental change (Frankham *et al.* 2002). Therefore, estimating and evaluating genetic diversity is a routine analysis in molecular ecology and especially conservation genetics. However, estimates of genetic diversity vary depending on the molecular marker and sampling approach used. For the giant panda, the applications of protein polymorphisms, mtDNA, minisatellites (DNA fingerprinting), microsatellites and single nucleotide polymorphisms (SNPs) have led to a wide array of data and a wide range of parameter estimates that render an understanding of panda genetic diversity problematic.

The first population genetic study used protein electrophoresis (Su *et al.* 1994) and showed only one locus to be polymorphic, suggesting a very low level of genetic diversity. Subsequently, mtDNA and DNA fingerprinting were applied (Fang *et al.* 1997; Zhang *et al.* 2002). Using a DNA fingerprinting probe, Fang *et al.* (1997) also found low levels of genetic diversity within the Liangshan and Xiaoxiangling populations. Zhang *et al.* (2002) analysed variation in mtDNA D-loop sequences and also inferred low genetic variation. Taken together, these studies, although based on a single molecular marker type and small sample size, presented a consistent picture of low genetic diversity in giant pandas (Table 1).

However, with the development of microsatellite markers for giant pandas (Zhang *et al.* 1995, 2009; Lü *et al.* 2001; Shen *et al.* 2005; Wu *et al.* 2009) and the trend towards combining multiple molecular marker types, the picture of panda genetic diversity started to change during the 2000s. Lü *et al.* (2001) performed a multimarker study that applied mtDNA RFLP, D-loop sequencing, DNA fingerprinting and microsatellites and detected moderate levels of genetic diversity, with Qinling possessing the least variation. More recently, combining mtDNA and ten microsatellite markers, Zhang *et al.* (2007) assessed genetic variation across the five extant mountain populations based on a considerable sample size (115–159 individuals), revealing moderate-to-high levels of mtDNA and microsatellite diversity. They concluded that low genetic variation was unlikely to be a critical threat to this species. With the improvement of noninvasive genetic sampling (Zhang *et al.* 1994, 2006; Fang *et al.* 1996; Ding *et al.* 1998; Zhan *et al.* 2006), genetic studies in wild panda populations based on large-scale faecal collections were rapidly implemented, and these studies also detected relatively high levels of microsatellite and mtDNA diversity (Zhan *et al.* 2006; He *et al.* 2008; Hu *et al.* 2010a,b; Yang *et al.* 2011; Zhu *et al.* 2011b) (Table 1).

Most recently, the panda genome has provided a step change into our insights on panda genetic variability, revealing surprisingly high autosomal and coding region heterozygosity rates (Li *et al.* 2010). These values are 1.95 times higher than the rates estimated for the human genome. However, it should be noted that the sequenced individual 'Jingjing' was a captive individual whose parents were from different mountain populations (Liangshan and Minshan),

Table 1 Estimates of genetic diversity of giant pandas in the wild and in captivity

Reference	Molecular marker	Sample size	Mountain population	Key index of genetic diversity	Wild or captive
	Single marker				
Su *et al.* (1994)	40 allozymes or proteins	12	MS, QIO, LS	Hp = 0.008	both
Fang *et al.* (1997)	1 DNA fingerprint probe	15	LS, XXL	Ht = 0.64	wild
Zhang *et al.* (2002)	655–978 bp of mt DNA D-loop region	32	QIN, MS, QIO, LS	13 variable sites, 16 haplotypes	wild
Yan *et al.* (2006)	8 microsatellite loci	27	Wolong BC	H_O = 0.574, H_E = 0.62, MNA = 5.5	captive
	8 microsatellite loci	39	QIO	H_O = 0.581, H_E = 0.779, MNA = 9.8	wild
Zhan *et al.* (2006)	9 microsatellite loci	66	A part of MS	H_O = 0.625, H_E = 0.609, MNA = 5.4	wild
Wang *et al.* (2007)	25 microsatellite loci	13	Wolong BC	H_O = 0.453, H_E = 0.719, MNA = 5.24	captive
	25 microsatellite loci	21	Chengdu BC	H_O = 0.475, H_E = 0.696, MNA = 5.48	captive
	5 microsatellite loci	7	–	H_O = 0.514, H_E = 0.725, MNA = 3.8	wild
He *et al.* (2008)	13 microsatellite loci	33	A part of MS	H_O = 0.488, H_E = 0.68, MNA = 6.2	wild
	13 microsatellite loci	30	A part of QIO	H_O = 0.553, H_E = 0.819, MNA = 7.6	wild
Shen *et al.* (2009)	11 microsatellite loci	34	Wolong BC	H_O = 0.672, H_E = 0.666, MNA = 5.55	captive
	11 microsatellite loci	49	Chengdu BC	H_O = 0.671, H_E = 0.634, MNA = 5	captive
	11 microsatellite loci	31	A part of MS	H_O = 0.52, H_E = 0.694, MNA = 5.64	wild
	11 microsatellite loci	25	A part of QIO	H_O = 0.483, H_E = 0.803, MNA = 7.36	wild
Yang *et al.* (2011)	10 microsatellite loci	42	A part of MS	H_O = 0.686, H_E = 0.703, MNA = 5.9	wild
	Multiple markers				
Lü *et al.* (2001)	mtDNA RFLP	19	QIN, MS, QIO	8 variable sites, 5 haplotypes, π = 0.22	wild
	268 bp of mtDNA D-loop region	36	QIN, MS, QIO	16 variable sites, 17 haplotypes	wild
	2 DNA fingerprint probes	18	QIN, QIO	MAPD = 0.383 or 0.315	wild
	18 microsatellite loci	36	QIN, MS, QIO	H_O = 0.44, MNA = 3.7	wild
Zhang *et al.* (2007)	655 bp of mtDNA D-loop region	159	QIN, MS, QIO, LS, XXL	24 variable sites, 39 haplotypes, Hm = 0.943	wild
	10 microsatellite loci	115	QIN, MS, QIO, LS, XXL	H_O = 0.565, H_E = 0.642, MNA = 7.1	wild
Hu *et al.* (2010a,b)	655 bp of mtDNA D-loop region	42	LS	11 variable sites, 9 haplotypes, Hm = 0.7364	wild
	12 microsatellite loci	52	LS	H_O = 0.683, H_E = 0.592, MNA = 4	wild
Zhu *et al.* (2011b)	655 bp of mtDNA D-loop region	32	XXL	5 haplotypes, Hm = 0.532	wild
	655 bp of mtDNA D-loop region	21	DXL	5 haplotypes, Hm = 0.747	wild
	9 microsatellite loci	32	XXL	H_O = 0.704, H_E = 0.656, MNA = 4.556	wild
	9 microsatellite loci	21	DXL	H_O = 0.66, H_E = 0.634, MNA = 4.667	wild

mtDNA, mitochondrial DNA; RFLP, restriction fragment length polymorphism; Hp, mean heterozygosity for protein; Ht, mean heterozygous ratio; Hm, haplotype diversity for mtDNA; π, nucleotide diversity; H_O, observed heterozygosity; H_E, expected heterozygosity; MNA, mean number of allele per locus; MAPD, mean average per cent difference. QIN, Qinling Mountains; MS, Minshan Mountains; QIO, Qionglai Mountains; LS, Liangshan Mountains; DXL, Daxiangling Mountains; XXL, Xiaoxiangling Mountains; Wolong BC, Wolong Breeding Center; Chengdu BC, Chengdu Breeding Center.

potentially upwardly biasing the heterozygosity detected. Hence, genome sequences of other individuals or population-level genomic analysis are required to verify this result.

The captive breeding of giant pandas is regarded as a potentially important means of conserving this endangered species and retaining its genetic variation. Given the goal of establishing a self-sustaining breeding population, the genetic diversity of captive populations must be monitored as an important aspect of management. Yan *et al.* (2006) and Wang *et al.* (2007) have assessed genetic variation in the two largest captive populations using microsatellites, and both found relatively high genetic diversity although the level was

somewhat lower than the wild population (Table 1). Both studies suggested that the focus of captive breeding should be on pedigree management rather than the incorporation of new wild individuals. More recently, using representative captive samples and wild samples, Shen *et al.* (2009) revealed that the genetic variability of captive populations was indeed lower and instead made the case that input of new genetic material from wild pandas might be necessary in the near future.

Demographical history

It is often cited that the giant panda has experienced a historical population bottleneck that has resulted in its current endangered status. The fossil records show dynamic changes in fossil recovery rates (from low to high to low) in the early, middle/late Pleistocene, to the Holocene (Pei 1974), which may correlate with changes in population size (Wu 2002). In contrast, some researchers believe that increased human activity in the Holocene is more likely to have affected this species (He 1998). Both inferences seem plausible but have lacked verification.

Bayesian coalescent simulation approach, allowing alternative demographical hypotheses to be tested on historical population fluctuations (Beaumont 1999), has recently been applied extensively to giant panda studies and has provided fresh insights into its demographical history (Zhang *et al.* 2007; Hu *et al.* 2010a; Zhu *et al.* 2010b). For example, in the smallest and most isolated population (Xiaoxiangling), Zhu *et al.* (2010b) detected the signal of a strong and recent 60-fold population reduction, starting about 250 years ago. This event was postulated to have been a consequence of a known dramatic increase in the local human population facilitated by the use of non-native crops at the peak of the Qing Empire. Hu *et al.* (2010a) reconstructed the demographical history of the southernmost Liangshan population and found evidence for a population decline in the order of 95–96% during the last millennium, most likely to have been due to anthropogenic habitat loss. However, for the three larger populations (Qinling, Minshan and Qionglai), different population trajectories have been inferred and seem to have started several thousand years ago or even further back in the past (Zhang *et al.* 2007), but have not been as drastic as those in the southern populations. These studies reveal a consistent message that anthropogenic pressure has played a critical role in the recent population declines, emphasizing the importance of habitat and other protection measures in current conservation plans.

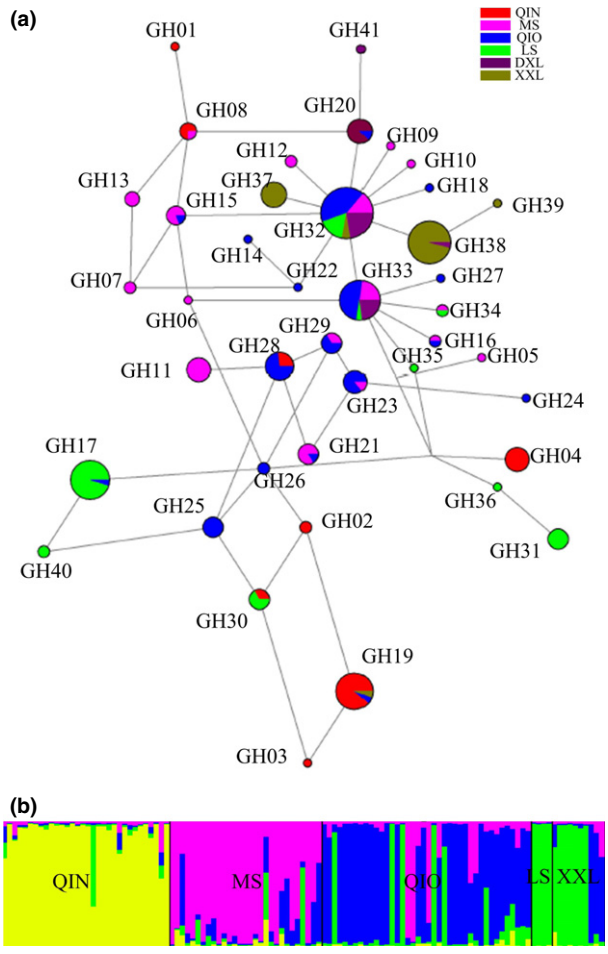


Fig. 3 (a) MtDNA network of giant pandas (data re-analysed from Zhang *et al.* 2007; Hu *et al.* 2010a; Zhu *et al.* 2011b); (b) microsatellite genetic structure of giant pandas (redrawn from Zhang *et al.* 2007). QIN, Qinling Mountains; MS, Minshan Mountains; QIO, Qionglai Mountains; LS, Liangshan Mountains; DXL, Daxiangling Mountains; XXL, Xiaoxiangling Mountains.

However, these studies have focused on detecting the most dominant signature in the data and have not attempted to reveal more detailed fluctuations during the giant panda's long evolutionary history (Qiu & Qi 1989). The application of Approximate Bayesian Computation (ABC) methods potentially allows multiple bottlenecks to be detected (e.g. Bertorelle *et al.* 2010), and the panda genome and re-sequencing efforts will enable the reconstruction of its population history with genome-level data.

Genetic structure and population differentiation

Under the pressure from anthropogenic habitat loss and fragmentation, wild populations of many mammals are facing population fragmentation and demographical isolation. Today, the giant panda occupies

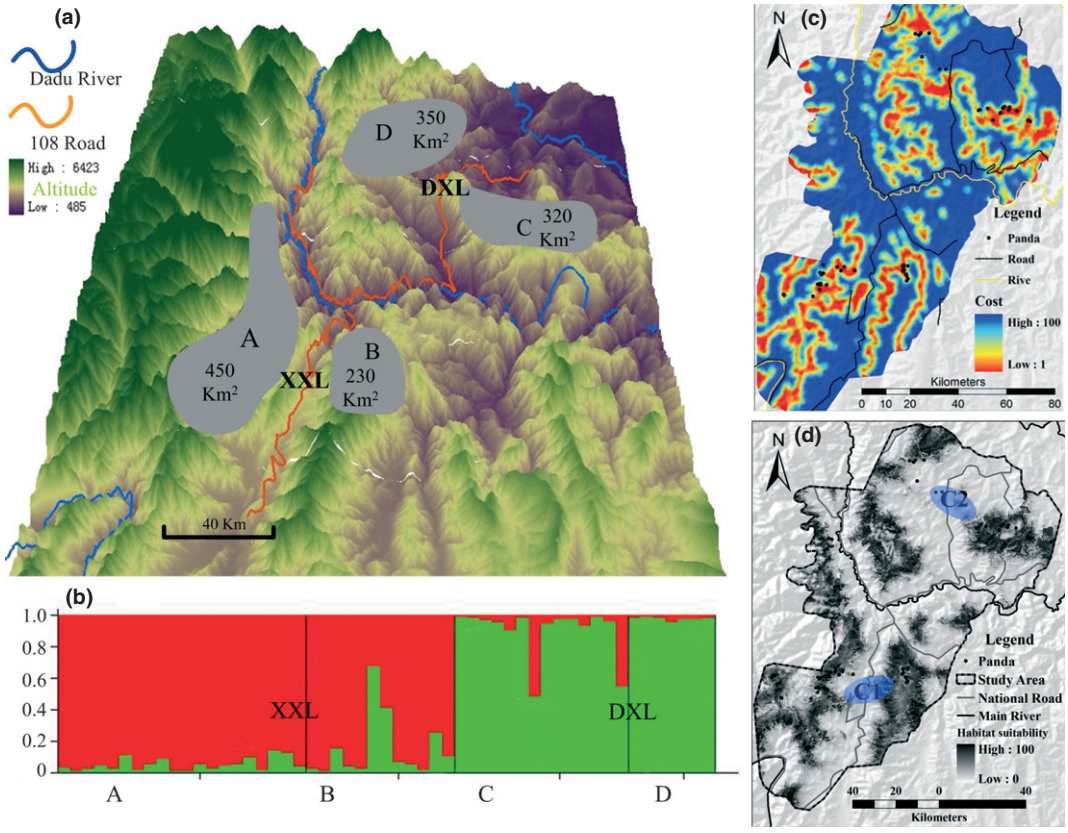


Fig. 4 A case study of landscape genetics on the most isolated and smallest populations of giant pandas in the Daxiangling (DXL) and Xiaoxiangling (XXL) Mountains (Redrawn from Zhu *et al.* 2010b, 2011b). (a) Study area; (b) genetic structure; (c) least cost path values using bamboo connectivity; (d) potential corridors between fragmented patches.

more than 20 habitat patches within six isolated mountain ranges (Loucks *et al.* 2001; State Forestry Administration 2006). It is therefore necessary to assess population genetic structure and disentangle underlying signals of short- and longer-term demographical processes to establish strategies to enable its long-term persistence.

Estimation of genetic differentiation between panda populations was first attempted by Fang *et al.* (1997) and Zhang *et al.* (1997). Using DNA fingerprinting, Fang *et al.* (1997) detected genetic differentiation between the Xiaoxiangling and Liangshan populations; however, in contrast, Zhang *et al.* (1997) found little differentiation among the Minshan, Qionglai and Liangshan populations using the mtDNA D-loop sequences. However, the combination of codominant nuclear markers and mitochondrial markers has made the picture clearer. Lü *et al.* (2001) found significant nuclear differentiation between Qinling and the other mountain populations, a result reconfirmed by Wan *et al.* (2003) using DNA fingerprinting and Zhang *et al.* (2007) using microsatellite markers (Fig. 3b). At a large spatial scale, mtDNA haplotypes do not segregate with geographical origin in giant pandas (Fig. 3a), but microsatellite frequencies are more distinct (Fig. 3b), hinting at the relatively recent demographical isolation of the Qinling population.

The causes driving population differentiation can be diverse, including natural barriers and anthropogenic habitat loss and fragmentation. In an attempt to answer this question for giant pandas, Zhu *et al.* (2011b) examined the neighbouring Xiaoxiangling and Daxiangling populations and found that genetic differentiation was mainly delineated by the Dadu River, although a well-established national highway could be seen to be precipitating further genetic divergence, revealing the relative effects of natural and anthropogenic barriers on gene flow (Fig. 4). In contrast, for the southernmost Liangshan population, fragmented by several county-roads, no significant genetic structure has been detected (Hu *et al.* 2010b). These studies suggest that effects of natural or anthropogenic barriers on gene flow may vary spatially, depending on landscape features (Short Bull *et al.* 2011) and factors such as fine-scale habitat suitability (Zhu *et al.* 2010a; Fig. 4).

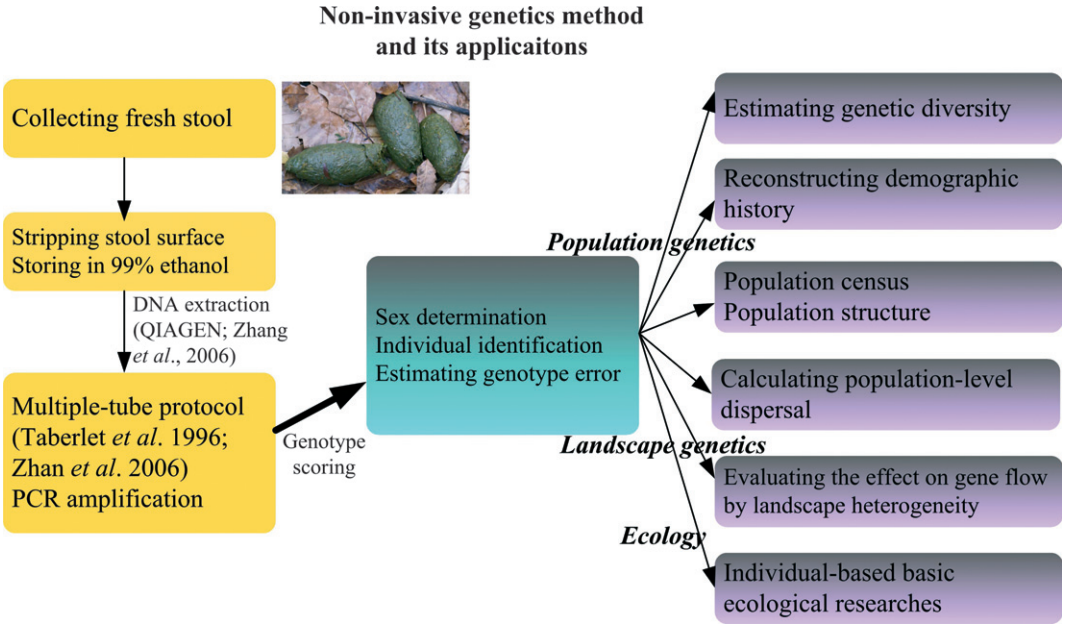


Fig. 5 Flowchart of noninvasive genetics method and its applications in the giant panda research.

Noninvasive genetics and its ecological application

Individual and sex identification

Accurate identification of giant panda individuals in the wild has proved to be a bottleneck for ecological research for decades, since it is difficult to observe and identify individuals because of their dense habitat and extreme wariness. Traditionally, an approach using bamboo bite length (measured indirectly through the size of bamboo stem fragments left in faeces) has been applied, a method originally developed to distinguish age groups (Schaller *et al.* 1985; Hu 1987). However, the precision of this approach was always known to be low. With the rapid development of noninvasive genetic sampling, researchers realized that as pandas defaecate over 120 faecal pellets per day, the noninvasive approach could be ideal for this species. The first noninvasive genetic sampling methods using hairs were developed by Zhang *et al.* (1994) (Box 1). Subsequently, classical DNA fingerprinting was also used to discriminate captive pandas (Fang *et al.* 1996). However, owing to the large amount of DNA required, the noninvasive application of this method is greatly limited. Zhan *et al.* (2006) proposed a more robust approach to individual identification based on microsatellite profiles amplified from faecal DNA samples of giant pandas. This method has helped to accelerate individual identification and has been widely applied in population studies during the last 5 years (Fig. 5; He *et al.* 2008; Hu *et al.* 2010b; Zhu *et al.* 2010b; Yang *et al.* 2011; Zhang *et al.* 2011).

Compared with the difficulties in identifying individuals, it has proved to be relatively easy to molecularly sex pandas with *Sex Determining Region Y* (*SRY*) gene. In giant pandas, *SRY* primers were developed to noninvasively sex faecal samples (Zhan *et al.* 2006). However, the *SRY* system produces only male-specific amplicons, and nonamplification can originate either from the presence of a female or PCR failure, so an X-Y homologous zinc finger protein gene (*ZFX*/*ZFY*) fragment is also amplified with *SRY* (Zhan 2006). Furthermore, depending on different primer combinations, the partially homologous *ZFX*/*ZFY* (Durnin *et al.* 2007; Xu *et al.* 2007) and amelogenin gene (*AMELX*/*AMELY*) (Xu *et al.* 2008) on both X and Y chromosomes of giant pandas have also been developed.

Population census

Molecular approaches developed for individual identification provide an opportunity to more accurately census wild populations. In the first application of this approach to giant pandas, Zhan *et al.* (2006) comprehensively collected 301 fresh faecal samples in a key panda reserve (Wanglang) and made a molecular census of 66–72 individuals, more than doubling the previous estimate of 27 individuals, suggesting the underestimation of traditional population census.

However, genotyping errors because of low quantity and/or quality of DNA in noninvasive samples remain a major challenge to the routine use of this method. The causes and consequences of genotyping errors, and potential solutions, have been reviewed by Pompanon *et al.* (2005). One important strategy of obtaining reliable genotypes is to perform multitube amplifications (Taberlet *et al.* 1996), although the precise method needed to produce statistically valid outcomes can be modified (e.g. Valière *et al.* 2002). The methods used by Zhan *et al.* (2006) were subsequently criticized because significant genotyping error would be expected to artificially inflate population size estimates (Garshelis *et al.* 2008; but see Zhan *et al.* 2009). To assess the magnitude of this error and its expected impact on genotyping studies, Zhan *et al.* (2010) developed a mathematical method to estimate genotyping error (i.e. allele dropout and false allele) rates applicable to the multitube approach, which showed that the error rate for the data set of Zhan *et al.* (2006) could have been maximally 9×10^{-4}, a rate that would have produced at most four erroneous genotypes.

Population-level dispersal

Owing to the panda's elusive nature, its dispersal is difficult to observe directly in the wild (Hu *et al.* 1985). However, with the help of large-scale noninvasive sampling and spatial genetic analysis, it can be explored at the population level (Zhan *et al.* 2007; Hu *et al.* 2010b). Zhan *et al.* (2007) analysed the spatial structure of related individuals and found a female-biased dispersal pattern at a fine scale in Wanglang Nature Reserve, in contrast to the male-biased dispersal pattern of many mammals (Lawson Handley & Perrin 2007). To verify this, Hu *et al.* (2010b) used spatial autocorrelation analysis and reconfirmed female-biased dispersal at a mountain-range scale in Liangshan. Several hypotheses have been proposed to explain this pattern, such as competition for key resources (birthing dens), inbreeding avoidance and asymmetry in breeding costs between the sexes (Zhan *et al.* 2007). However, more ecological studies are required to test these hypotheses on the ground.

Other important applications

Molecular-based individual and sex identification for wild pandas not only helps facilitate accurate population census, but has wider applications in other ecological studies, such as those focusing on habitat selection, reproductive ecology and conservation medicine. Applying molecular sex determination, for instance, Qi *et al.* (2011) detected significant gender differences in habitat use in wild pandas and Nie *et al.* (2012) found sex differences in scent-marking patterns. Using molecular individual identification, Zhang *et al.* (2011) analysed faecal parasite loads in wild panda faeces across the six mountain populations and found that statistical differences between mountains were artificially inflated when individual identity was not accounted for in the model. Therefore, applying noninvasive genetics has provided not only deep insights into the ecology of pandas but also important information for conservation management such as reintroduction or translocation, which could not have been achieved using traditional methods. This approach will continue to enhance studies of panda ecology and conservation, especially since DNA profiling is now incorporated into the ongoing Fourth National Survey of Giant Pandas.

Genetic basis of giant pandas adaptation

An adult panda consumes a remarkable average of 12.5 kg of bamboo daily (Hu *et al.* 1985). However, because it lacks the long digestive tract of typical herbivores, extensive gut-based fermentation is impossible (Dierenfeld *et al.* 1982). The physiological and genetic basis of the panda's adaptation to its bamboo diet has therefore been of interest to scientists for decades. Through the panda genome (Li *et al.* 2010), an understanding of the genetic basis of mechanisms underpinning adaptations to the bamboo diet can be attempted. Some remarkable insights have already been made.

Digestion of cellulose and hemicellulose in bamboo

Giant pandas can digest not only protein and fat, but also partially digest hemicelluloses and celluloses (Dierenfeld *et al.* 1982). Because the panda genome lacks the enzyme homologues needed for cellulose digestion (Li *et al.* 2010), digestion of these bamboo fibres must be dependent on gut microflora. Previous studies on the panda gut microbiome have identified the presence of three predominant bacteria – *Escherichia coli*, *Streptococcus* and Enterobacteriaceae; however, none of them are known to aid cellulose digestion (Zhang *et al.* 1988; Hirayama *et al.* 1989; Wei *et al.* 2007). Ley *et al.* (2008) used a large data set of prokaryotic ribosomal RNA gene sequences to investigate the evolution of mammals and their gut microbes (with one faecal sample of a captive panda included) and detected a high proportion of Firmicutes in the panda's gut microbiome.

Using faecal samples collected in the wild and captivity, Zhu *et al.* (2011a) performed a metagenome analysis and examined over 5000 prokaryotic ribosomal RNA gene sequences. They detected not only lower species richness within the gut microbiome, but

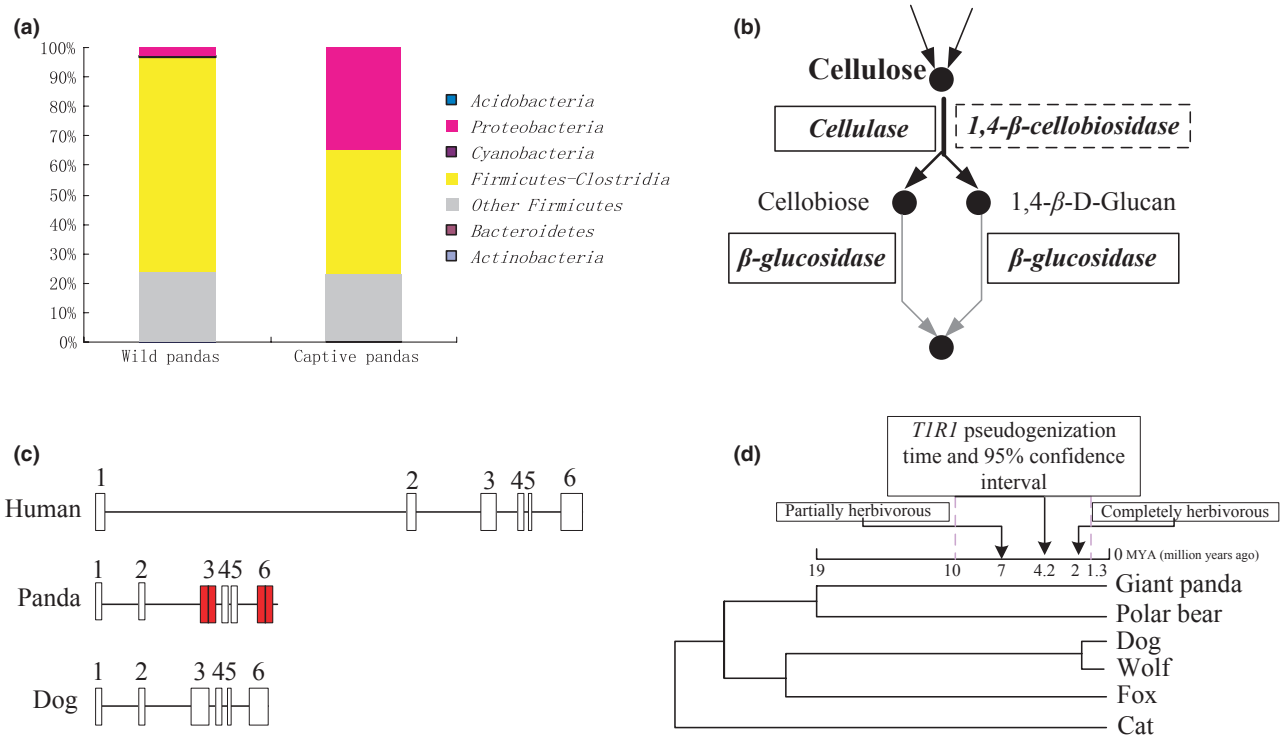


Fig. 6 Genetic basis of adaptation in giant pandas. (a) Gut microbial flora of giant pandas (redrawn from Zhu *et al.* 2011a); (b) pathway of bamboo cellulose digestion by gut microbes in giant pandas; (c) structure of the umami receptor T1R1 gene in human, giant panda and dog (redrawn from Li *et al.* 2010); (d) posterior probability distributions of T1R1 pseudogenization date (redrawn from Zhao *et al.* 2010).

found seven operational taxonomic units (OTUs) unique to pandas among 13 OTUs closely related to microbes known to digest cellulose, Clostridium groups I and XIVa (Fig. 6a). Metagenomic analysis identified not only putative genes coding cellulose and hemicellulose-digesting enzymes in the gut microbiome (Fig. 6b), but also the lowest abundance of cellulases and endohemicellulases (2%) compared with other herbivores and omnivores. They concluded that 'the presence of putative cellulose-digesting microbes, in combination with adaptations related to feeding, physiology and morphology, shows that giant pandas have evolved a number of traits to overcome the anatomical and physiological challenge of digesting a diet high in fibrous matter'.

Genetic consequence of the dietary switch to bamboo

The taste senses comprise sweet, salt, sour, bitter and umami, the latter of which is now considered to be an important factor in the development of diet (Jiang *et al.* 2012). Umami perception occurs through components of meat and other protein-rich foods. Research has revealed that the molecular basis of umami receptor involves the T1R1/T1R3 heterodimer (Chandrashekar *et al.* 2006). The panda genome revealed that the critical T1R1 gene has become pseudogenized, suggesting that the loss of function of the T1R1 gene may have contributed to the panda's dietary switch (Li *et al.* 2010; Fig. 6c). To test this hypothesis, Zhao *et al.* (2010) sequenced all six T1R1 exons and confirmed the pseudogenization of this gene. Further, they estimated this pseudogenization occurred to be about 4.2 million years ago, which matches the approximate date of the panda's dietary switch (Fig. 6d). To address the aforementioned question, beyond taste receptor genes, Jin *et al.* (2011) analysed 166 major genes involved in the 'appetite-reward system' and found a deletion in the catechol-o-methyltransferase (COMT) gene, which is probably to result in loss of function in its catecholamine metabolic pathways. This finding suggests that the dopamine metabolic system is probably not competent in the panda, suggesting unusual metabolic processes may govern the species' food choice.

In addition, the subcellular distribution of alanine glyoxylate aminotransferase (AGT) is thought to be related to dietary choice in many vertebrates (Ichiyama *et al.* 2000). AGT activity tends to be mitochondrially located in carnivores, peroxisomal in herbivores, and both mitochondrial and peroxisomal in omnivores

(Danpure *et al.* 1990). Birdsey *et al.* (2005) examined the relationship between AGT distribution and diet in 77 mammalian species and found a highly significant correlation between AGT distribution and diet, independent of phylogeny, indicating a response to episodic changes in dietary selection pressure. As expected, in contrast to other carnivores, AGT targeting in pandas is more peroxisomal and less mitochondrial (Birdsey *et al.* 2004).

Although these studies highlight some interesting findings on the evolutionary consequences of the panda's dietary switch, the causal mechanism is not yet understood, and we still do not know why pandas initially changed their diet.

Adaptive evolution of disease resistance genes

Measuring levels of polymorphism at major histocompatibility complex (MHC) genes can provide indirect measures of the immunological adaptation and fitness of populations (Ujvari & Belov 2011). In panda populations, relatively modest levels of variation at MHC genes have been observed at class II MHC genes including DRB, DQA and DQB, and classical balancing selection mechanisms have been inferred in the maintenance of genetic variability in these genes (Wan *et al.* 2006; Zhu *et al.* 2007; Chen *et al.* 2010). For class I genes, one study has identified three genes from a bacterial artificial chromosome library of giant pandas (Pan *et al.* 2008), but these have not yet been assayed for variation in natural populations. To date, studies have mainly focused on quantifying diversity; however, no study focusing on the interaction of MHC genetic variation with disease resistance has been conducted. Research is ongoing, to examine the relationship between MHC class II genes and parasite load in wild pandas, which could have important implications for captive breeding programs and conservation management.

Conservation implications

The giant panda is listed by IUCN as endangered C2a (i), defined as follows: *Population size estimated to number fewer than 2500 mature individuals with a continuing decline, observed, projected, or inferred, in numbers of mature individuals and no subpopulation estimated to contain more than 250 mature individuals*. The implications of the Zhan *et al.*'s (2006) findings for estimates of total population size (and by extension estimates per mountain range) could only be extrapolative when published and a more precise population estimate (total and per mountain range) will hopefully be resolved by the ongoing National Survey. However, as it has now been established that giant pandas do not have lower genetic diversity than expected, given their demographical history (Zhang *et al.* 2007; Li *et al.* 2010), we can be reasonably sure that genetic variation is not the critical factor responsible for its endangered status and conservation priority should now be given to habitat protection and restoration to provide sufficient carrying capacity for future population expansion.

In conservation genetics, information on patterns of population genetic differentiation and the processes giving rise to these are often used in helping to determine management units and to guide conservation and management actions (Bertorelle *et al.* 2009). A number of studies of population genetic structure in giant pandas have shown that the Qinling population is significantly differentiated and is demographically independent from the populations in Sichuan (Lü *et al.* 2001; Wan *et al.* 2003; Zhang *et al.* 2007), establishing its unique genetic status. Therefore, the Qinling population has been recommended as a distinct management unit (or even as a subspecies, Wan *et al.* 2005), whereas other mountain populations have been suggested to be a single management unit (Zhang *et al.* 2007). It is important to note, however, that this may change as more genetic data become available. Differentiation among populations at genetic systems underpinning adaptive evolution may provide contrasting results and the gut microbiome in different mountain populations might be even more discordant (Zhu *et al.* 2011a).

Understanding effects of landscape features on gene flow has important implications for guiding corridor construction. Landscape genetics studies have showed that large rivers and, increasingly, major roads have inhibited and/or are inhibiting dispersal and gene flow among panda populations (Hu *et al.* 2010b; Zhu *et al.* 2011b). Further, anthropogenic habitat loss and fragmentation are eroding panda habitat, affecting dispersal and gene flow (Hu *et al.* 2010b; Zhu *et al.* 2010a). These findings suggest that, to maintain the persistence of wild populations, habitat corridors should be re-established to allow dispersal and avoid the formation of isolated populations (Qi *et al.* 2012). In this context, it is noteworthy that genetic studies have recently been cited as compelling evidence in Chinese government plans to construct habitat corridors in the Xiaoxiangling and Daxiangling Mountains (Zhu *et al.* 2011b).

Translocation and reintroduction can be an important means of recovering wild populations of endangered species. Giant pandas have become progressively divided into six isolated mountain populations, and subdivision is ongoing as habitat fragmentation spreads (State Forestry Administration 2006). As a result, some subpopulations are facing the threat of local extinction. For example, the smallest and most isolated population in the Xiaoxiangling Mountains has been predicted to

have a high probability of local extinction in the near future (Zhu *et al.* 2010b, 2011b). So, to assist this small population to recover, a wild female panda was successfully translocated into this region in April 2009 by the Chinese government and survives until today.

Future directions

The future application of genetics in giant panda conservation and research is probably to be focused on applied management actions (Swaisgood *et al.* 2011). As such the first question that needs to be asked is 'what do we really need to know?' Below we list a series of questions, in no order of priority, to which genetics is probably to be applied.

First, we need to apply the noninvasive genetic methods already developed to enhance the Fourth National Survey of Giant Pandas, so that molecular censusing can be used to augment conventional ecological population estimates and to further document the partitioning of genetic variation across its whole range. Here, the major challenge is the scale of the analysis, and it seems probably that no single laboratory will be able to complete the molecular survey independently. Therefore, participating laboratories need to ensure that methods are standardized to ensure comparability of DNA profiling results. An important choice implicit in this effort is the genotyping method to be used. The genome sequence now allows all genetic markers to be applied, and it may be easier in the future to exploit SNP typing, rather than microsatellites. The ability to interrogate thousands of SNPs from faecal DNA would further transform ecological, evolutionary and conservation studies in this species. Genome re-sequencing would also be an appropriate strategy; however, the reliability of this method for faecal DNA remains to be tested.

Second, as captive breeding, reintroduction and translocation are issues that will continue to be of importance in panda management, DNA profiling using neutral markers and genes of known adaptive significance should be screened in relevant individuals. In this context, the captive populations need complete genetic characterization, including assigning individuals of uncertain geographical origin to their natal populations, so that the two management units or subspecies identified previously can be managed effectively.

Third, it is important to establish as far as possible the extent of adaptive differentiation between the Qinling management unit and the remaining populations. It is already known that a brown pelage phenotype, which is not found in Sichuan so far, is prevalent in Qinling, and the habitat is different between these ranges. Ongoing efforts to establish general phylogeographical patterns and processes for a variety of endemic organisms in the region (e.g. Zhan *et al.* 2011), including the bamboo species that make up the panda's diet, will further underpin this process. The analysis required to accurately document this adaptive differentiation could now include whole genome and transcriptome re-sequencing. However, discriminating the signals of natural selection from demographical processes remains challenging, and it is important to validate genome scan results using functional tests, although this validation may be difficult in endangered species. Additionally, monitoring the response of individuals in both management units to climatic episodes that limit bamboo availability will also help understand adaptive responses in this species when bamboos undergo periodic flowering and die off.

Fourth, a more detailed understanding of basic demographical processes in panda populations will allow better predictive models to be constructed to assist conservation management plans. For instance, what is the level of reproductive skew among male pandas? Understanding the determinants of male effective population size may allow not only better model parameterization but also more judicious translocation practices among isolated populations where the potential genetic gain of artificially assisted gene flow can depend on relative reproductive success.

Finally, and to return to question posed at the end of our introduction, how is the status of this species linked to its evolutionary peculiarities? Although this might seem at first glance to be a question of little conservation relevance, there are important reasons to study it. Questions such as how did giant pandas switch to their present diet and what evolutionary processes were involved, what is the adaptive relevance of the brown pelage phenotype and why is it nonrandomly distributed across the range, how flexible is the giant panda's diet *in extremis*, and many others can be applied to better conserve this species for future generations, because when we understand these processes better, we may be able to cater for future scenarios for this unique species and its habitat in a more informed way.

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