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Sorting Out the Genetic Background of the Last Surviving South China Tigers

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Abstract

The South China tiger (*Panthera tigris amoyensis*) is endemic to China and also the most critically endangered subspecies of living tigers. It is considered extinct in the wild and only about 150 individuals survive in captivity to date, whose genetic heritage, however, is ambiguous and controversial. Here, we conducted an explicit genetic assessment of 92 studbook-registered South China tigers from 14 captive facilities using a subspecies-diagnostic system in the context of comparison with other voucher specimens to evaluate the genetic ancestry and level of distinctiveness of the last surviving *P. t. amoyensis*. Three mtDNA haplotypes were identified from South China tigers sampled in this study, including a unique *P. t. amoyensis* AMO1 haplotype not found in other subspecies, a COR1 haplotype that is widespread in Indochinese tigers (*P. t. corbetti*), and an ALT haplotype that is characteristic of Amur tigers (*P. t. altaica*). Bayesian STRUCTURE analysis and parentage verification confirmed the verified subspecies ancestry (VSA) as the South

China tiger in 74 individuals. Genetic introgression from other tigers was detected in 18 tigers, and subsequent exclusion of these and their offspring from the breeding program is recommended. Both STRUCTURE clustering and microsatellite-based phylogenetic analyses demonstrated a close genetic association of the VSA South China tigers to Indochinese tigers, an issue that could only be elucidated by analysis of historical South China tiger specimens with wild origin. Our results also indicated a moderate level of genetic diversity in the captive South China tiger population, suggesting a potential for genetic restoration.

Subject areas: Conservation genetics and biodiversity, Population structure and phylogeography
Key words: admixture, South China tiger, inbreeding, microsatellite, mtDNA

Introduction

The tiger (*Panthera tigris*) is one of the largest felids in the world and a widely recognized flagship species of wildlife conservation. It is commonly accepted that there are 6 living subspecies of the tiger, including the Bengal tiger (*P. t. tigris*), Amur tiger (*P. t. altaica*), South China tiger (*P. t. amoyensis*), Sumatran tiger (*P. t. sumatrae*), Indochinese tiger (*P. t. corbetti*), and the Malayan tiger (*P. t. jacksoni*) (Luo et al. 2004, 2006, 2008, 2010a, 2010b; Goodrich et al. 2015; Liu et al. 2018). However, based on primarily morphological and ecological data, Wilting et al. (2015) and Kitchener et al. (2017) proposed a revised tiger intraspecific taxonomy to recognize only 2 subspecies, or *P. t. sondaica* including tigers from the Sunda Islands of Sumatra, Java, and Bali, and *P. t. tigris* merging all tiger populations from continental Asia.

The South China tiger was first described in 1905 by Max Hilzheimer, a German zoologist, based on 5 specimens collected in Hankau (now Hankou, Hubei Province, China). He described that the South China tiger is similar in height to the nominate Bengal tiger, but differs in skull. Relative to *P. t. tigris*, *P. t. amoyensis* has shorter carnassials and molars, a lighter and more yellowish coat with more sharp-edged and narrower stripes, and paler paws, face, and abdomen. In addition, the cranial region of *P. t. amoyensis* is shorter with orbits set closer together and the postorbital processes are larger than that of *P. t. tigris* (Hilzheimer 1905). Ecological analysis (Wilting et al. 2015) also indicated a low niche overlap of the South China tiger with other subspecies. Moreover, genetic analyses consistently placed the mtDNA haplotype uniquely found in *P. t. amoyensis* (AMO1, Luo et al. 2004) as basal in the tiger mitochondrial phylogeny (Wilting et al. 2015; Xue et al. 2015; Liu et al. 2018). All these data suggested an evolutionary distinctiveness of the South China tiger and its validity as a subspecies.

The South China tiger was once widely distributed in China, spanning about 2000 km from east to west and 1500 km from north to south (Liu and Yuan 1983; Lu and Sheng 1986) (State Forestry Administration, unpublished data). Historically, the northern most distribution was recorded in the Qinling Mountain and Yellow River Basin at about 35°N and its southern most range extended to Guangdong, Guangxi and Yunnan Provinces around 21°N (Lu and Sheng 1986) (State Forestry Administration, unpublished data). It was estimated that the South China tiger population numbered more than 4000 during the 1950s (Tan 1987). Unfortunately, large-scale "pest" eradication campaigns against wild tigers combined with habitat loss have led to a precipitous decline and eventual collapse of its wild population (Tilson et al. 2004). No living South China tiger has been sighted in the wild for the past 3 decades and it is listed in the IUCN Red List of Threatened Species as "Critically Endangered (Possibly Extinct in the Wild)" (Tilson et al. 2004; Nyhus 2008; Qin et al. 2015).

Among all the tiger subspecies, *P. t. amoyensis* perhaps is the most taxonomically controversial, as it has only survived by a captive population with unresolved genetic heritage and it is likely affected by accidental admixture and erroneous records during the captive management. The Chinese Association of Zoological Gardens has implemented a coordinated South China tiger captive breeding and management program since 1994, beginning with 47 tigers including 27 males and 20 females in the Studbook. Now the population is maintained by 14 zoos or breeding facilities throughout China, representing the last hope to preserve and recover this vanishing lineage of tiger. By November 2016, there were 144 living tigers in the captive program, all recorded as descendants of 6 wild founders between 1958 and 1970, 1 female from Fujian Province in southeast China and 5 (2 males and 3 females) from Guizhou Province in southwest China (Yin 2016). In fact, a total of 18 tigers were captured from the wild in southern China and housed in captivity from 1955 to 1970, but only 6 of them ever produced progeny (Figure 1; Table 1). As most individuals are derived from a small number of founders, the current captive South China tigers might suffer from low levels of genetic diversity and high levels of inbreeding (Wei et al. 2005; Xu et al. 2007; Yin 2016). Furthermore, because large numbers of breeding and pedigree records went missing for the period between the 1960s and 1980s, it is suspected that some individuals in the captive program might be of uncertain ancestry due to accidental introgression of tigers from other sources rather than the South China tiger.

The first genetic analysis of *P. t. amoyensis* was conducted by Luo et al. (2004), which included 5 specimens (studbook #242, #256, #258, #129, and #141) collected from 2 Chinese zoos in the mid-1990s. Two distinctive lineages were revealed based on mtDNA and microsatellite evidence: one from the Chongqing Zoo that is unique and distinct from the other subspecies, and another from the Suzhou Zoo that is closely associated with the mainland Indochinese tiger. Subsequently, partial mitochondrial ND5 was sequenced in 46 captive *P. t. amoyensis* (Wei et al. 2005) and the full mitogenome sequence was obtained from 2 individuals of *P. t. amoyensis* (Zhang et al. 2011). Both studies affirmed the divergence within the captive population of *P. t. amoyensis*.

According to the studbook (Yin 2016), the 2 separate lines have been cross-bred since 1995 to minimize potential inbreeding depression. Thus, the likelihood of identifying a substantial number of unique *P. t. amoyensis* from present captive population is presumably slim. In addition, owing to the lack of definitive morphological markers distinguishing tiger subspecies (Kitchener 1999; Kitchener and Yamaguchi 2010; Wilting et al. 2015; Kitchener et al. 2017)

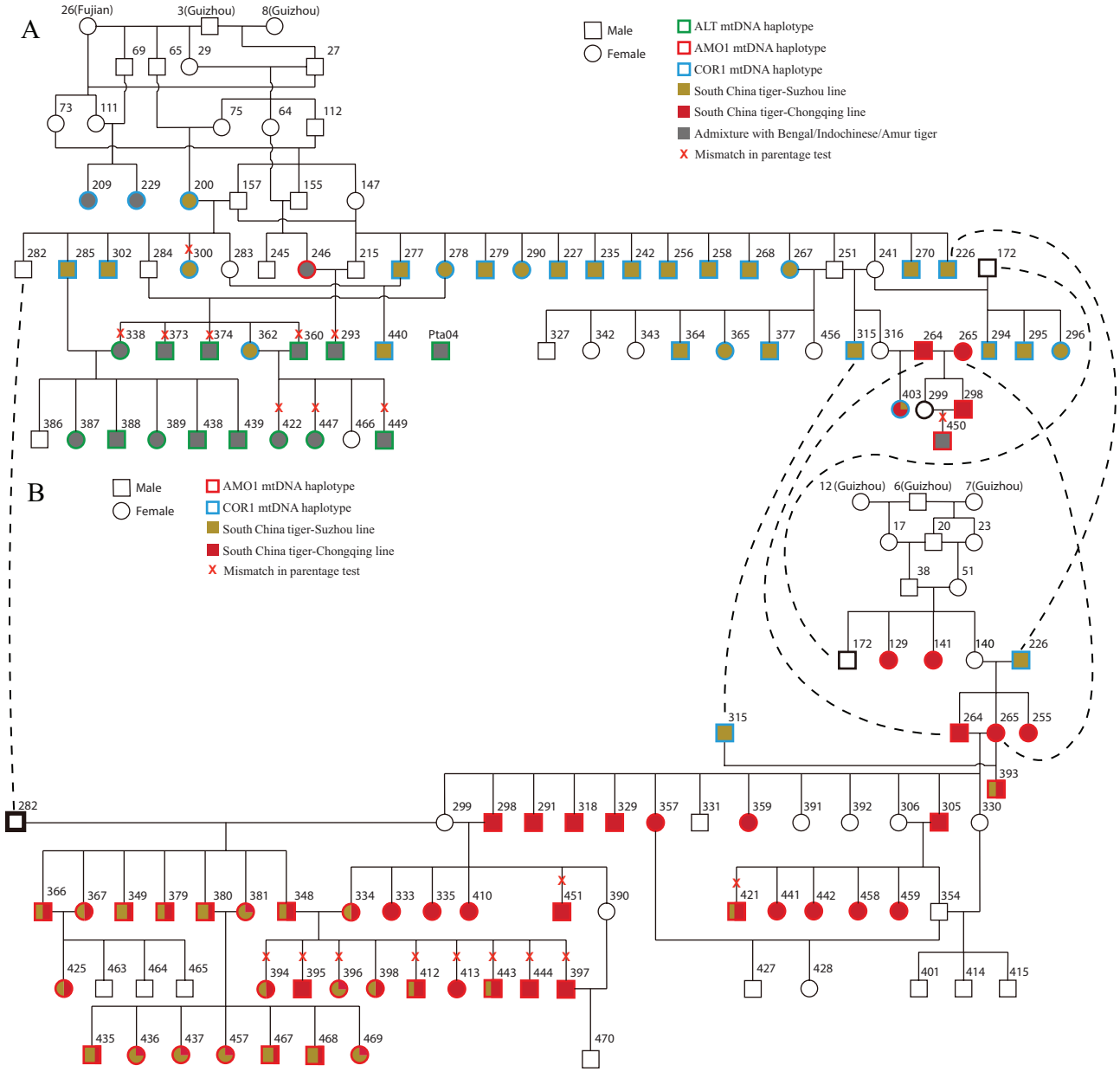

Figure 1. Pedigree of the 2 major lines forming the captive South China tiger population in China and genetic ancestry of each individual based on mtDNA haplotype and microsatellite composite genotypes from 30 loci. All living South China tigers in captivity as of 18 September 2010 are included in the pedigree, with their genealogies illustrated and studbook numbers labeled. Three mtDNA haplotypes were identified from the captive population, including a unique *P. t. amoyensis* AMO1 haplotype not found in other subspecies, a COR1 haplotype that is widespread in Indochinese tigers (*P. t. corbetti*), and an ALT haplotype that is characteristic of Amur tigers (*P. t. altaica*). Colors of the area charts correspond to biparental subspecies ancestry composition based on STRUCTURE clustering analysis (more details can be found in Supplementary Table S1). Crosses indicate parentage mismatches detected within the pedigree. Mitochondrial and microsatellite assignments confirmed the VSA as the South China tiger in 74 individuals. (**A**) The Suzhou line of captive South China tigers includes 18 individuals (filled in gray) with genetic introgression from Indochinese, Amur, or Bengal tigers, probably introduced by mistake, and are recommended to be purged from the coordinated captive breeding program. Such mistakes are evident in mother–offspring mtDNA haplotype mismatches and discrepancies in microsatellite genotypes. (**B**) All individuals from the Chongqing line of captive South China tigers carry the *P. t. amoyensis*-unique mtDNA haplotype AMO1. The 2 lines were managed separately until mid-1990s, and the dashed lines indicate the cross-bred individuals between Suzhou and Chongqing lines. See online version for full color.

and possible erroneous breeding records during captive husbandry, inadvertent admixture from other subspecies into the South China tiger captive population is hard to detect. To this end, an explicit genetic assessment of the captive tigers in China in comparison with other voucher subspecies is urgently needed to provide the critical information that is fundamental for improved conservation management and to validate the uniqueness or non-uniqueness of current *P. t. amoyensis*.

In this study, we collected the largest ever sample set from the coordinated captive population throughout China and applied the verified subspecies ancestry (VSA; Luo et al. 2008, 2010a, 2010b) molecular diagnostic system to examine the genetic composition of

the last living *P. t. amoyensis*. The results illuminate the genetic structure of the extant population and shed light on the potential to eventually reintroduce captive-born tigers to their original habitat in the long term (Fàbregas et al. 2015; Qin et al. 2015).

Table 1. Wild-caught tigers recorded in the South China tiger captive management in China

	Studbook No.	Sex	Birth date	Capture date	Capture location
Founder	3	Male	~1956	~1959	Keuiyang, Guizhou
	6	Male	~1958	~1958	Qingzhen, Guizhou
	7	Female	~1958	~1958	Changshun, Guizhou
	8	Female	~1958	~1962	Zunyi, Guizhou
	12	Female	~1959	~1959	Bijie, Guizhou
	26	Female	~1967	~1970	Fuchou, Fujian
Other individuals from wild	1	Female	~1955	~1955	Sichuan
	2	Male	~1955	~1959	Qingzhen, Guizhou
	4	Male	~1956	~September 1956	Wuhan, Hubei
	5	Female	~1957	~1959	Guizhou, Guizhou
	9	Female	~1958	~1959	Zunyi, Guizhou
	10	Male	~1958	~1959	Guangdong
	11	Female	~1958	~1959	Guangdong
	13	Male	~1959	~1962	Guizhou
	14	Male	~1959	~1959	Guangshun, Guizhou
	15	Female	~1959	~1959	Keuiyang, Guizhou
	16	Female	~1960	~1960	Canton Ch, Guangdong
	25	Female	~1966	~1966	Hunan

Materials and Methods

Samples

Eighty-seven captive South China tigers were sampled for blood from 14 zoos or breeding centers between 2005 and 2009. This sample set covered over 75% of the entire population (N = 98) during the time of collection and included all pedigrees derived from the 6 founders recorded in the studbook (Figure 1A,B). Genomic DNA from blood was isolated using a standard proteinase K digestion and phenol–chloroform extraction procedure (Sambrook et al. 1989). The microsatellite and mitochondrial data of 108 voucher tigers (Luo et al. 2004) were used as the reference data set. The reference panel of voucher specimens included 34 Amur, 30 Indochinese, 22 Malayan, 6 Bengal, and 16 Sumatran tigers. Data from the 5 South China tigers used by Luo et al. (2004) were also included, making a total of 92 captive South China tigers in the analysis.

Mitochondrial DNA Analysis

Ten PCR primers amplifying a total of 4687 bp of cytoplasmic mtDNA sequence were applied in this sample set following procedures described previously (Luo et al. 2004). The concatenated mtDNA haplotype included *ND5* (C53F1/T598R, C708F/T1300R), *ND6* (C1494F/T1936R), *CytB* (C2339F/T2893R), *CR* (CR-UPF/CR-R2B), *12S* (C-12S-F/N/C-12S-R), *ND1* (C8276F/T8620R), *ND2* (T8942F/C9384R, C9366F/T9882R), and *COI* (C11020F/T11428R), which encompassed 46 subspecies-diagnostic or -specific sites that distinguished all 6 living tiger subspecies. Sequences were unambiguously aligned using BioEdit and visually inspected to assign mtDNA haplotype in reference to voucher subspecies data set (GenBank accession numbers AY736559–AY736808).

Microsatellite Analysis

The same 30 polymorphic microsatellite loci used for VSA assignment of generic tigers were genotyped in captive South China tigers following previously published procedures (Luo et al. 2004). Three samples (Pti-88, Pti-212, and Pti-270; Luo et al. 2008) that have been used in the VSA panel served as between-run calibration, so that microsatellite data generated in this study were compatible with voucher tiger genetic profiles.

Possible null alleles, allele dropout, and scoring errors owing to stutter peaks of microsatellite genotypes were checked in MICROCHECKER v. 2.2.3 (Van Oosterhout et al. 2004). MICROSAT (Minch et al. 1995) was used to analyze microsatellite genetic variation in terms of average observed and expected heterozygosity, average number of alleles per locus, average allele size per locus, number of unique alleles, and average variance. A pairwise genetic distance matrix among individual tigers, based on the proportion of shared alleles (Dps) or kinship coefficient (Dkf) with the [1 – ps/kf] option in MICROSAT (Minch et al. 1995), was used to construct a neighbor-joining (NJ) tree of individuals using the program NEIGHBOR in PHYLIP 3.5 (Felsenstein 1989; as in Luo et al. 2004). Bootstrapping values of 100 replicates were obtained in MICROSAT (Minch et al. 1995) to show the possibility of the individual-based microsatellite phylogeny. In addition, pairwise genetic relatedness (R_{XY}) values among individuals within a population were estimated in ML-RELATE (Kalinowski et al. 2006), which calculated the maximum likelihood of relatedness (R_{XY}) ranging from 0 (no relatedness between any pair of individuals in the population) to 1 (all individuals are genetically identical). The population inbreeding coefficient F_{IS} were analyzed through AMOVA (analysis of molecular variance) with 10 000 permutations in ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010).

Bayesian clustering analysis was run in STRUCTURE (Pritchard et al. 2000) to infer genetic structure without prior population information. The number of clusters (K) was set from 2 to 10, assuming an admixed ancestry and correlated allele frequency model. For each value of K, 10 independent simulations of 1 000 000 replications were performed after 50 000 burn-in steps and produced consistent results for the same value of K. Each run yielded a log-likelihood value (Ln probability) of the clustering scenario, and the delta K values were used to infer the best-fit number of population clusters. The likelihood (q) of an individual being assigned to a primary cluster was used to show the certainty that a tiger could be designated to one of the voucher subspecies or, alternatively, the extent of intersubspecies

Table 2. Mitochondrial DNA haplotypes in the South China tiger captive population

Location	MtDNA haplotype	Number of individuals	Studbook number
Changsha Zoo	AMO1	1	#396
	COR1	1	#270
Chengdu Zoo	AMO1	2	#412, #410
Chongqing Zoo	AMO1	3	#129[a], #141[a], #397
	COR1	3	#235, #227, #229
Fujian Meihuashan Institute of South China Tiger Breeding	ALT	12	#449, #447, #438, #439, #360, #338, #373, #387, #374, #388, #389, #422
	COR1	3	#285, #278, #362
Fuzhou Zoo	COR1	3	#302, #209, #290
Guangzhou Zoo	ALT	1	? (no studbook number)
	AMO1	1	#425
Jiujiang Zoo	COR1	1	#279
Xiamen Zhongshan Park	COR1	1	#268
Luoyang Wangcheng Garden	AMO1	10	#457, #291, #467, #468, #469, #380, #435, #381, #436, #437
	COR1	3	#200, #277, #440
Nanchang Zoo	AMO1	2	#246, #393
	COR1	2	#315, #403
Safari Park Guiyang	AMO1	2	#421, #413
Safari Park Shenzhen	AMO1	1	#335
Shanghai Zoo	ALT	1	#293
	AMO1	26	#366, #367, #305, #255, #348, #334, #349, #395, #398, #441, #450, #442, #443, #444, #451, #379, #394, #458, #459, #318, #265, #264, #329, #359, #357, #333
	COR1	1	#226
Suzhou Zoo	AMO1	1	#298
	COR1	11	#242[a], #256[a], #258[a], #226, #365, #364, #295, #294, #267, #300, #296, #377

[a]Data from Luo et al. (2004).

admixture. A tiger would be considered to have a single VSA if it is supported by both mitochondrial and microsatellite results ($q > 0.8$); an individual with an assignment discrepancy between matrilineal and nuclear genetic data, or with affiliations to 2 or more subspecies ($0.1 < q < 0.8$ each) based on microsatellite assignment test, would be classified as of admixed ancestry (Luo et al. 2010b).

Results

Mitochondrial DNA Haplotype Assignment

Three concatenated mtDNA haplotypes were detected from the 87 captive South China tiger specimens, corresponding to voucher subspecies haplotypes AMO1 (*P. t. amoyensis*, $N = 47$, 54% of the sampled captive population), COR1 (*P. t. corbetti*, $N = 26$, 30%), and ALT (*P. t. altaica*, $N = 14$, 16%) (Luo et al. 2004; Table 2; Figure 1A,B). It is worth noting that 12 of the 14 tigers carrying the Amur tiger mtDNA haplotype ALT were from the Meihuashan Institute of South China Tiger Breeding in Fujian (Table 2) and contributed to the breeding program until 2012. The other 2 tigers were originally from Guangzhou Zoo, one of which was transferred to Shanghai Zoo at the time of sampling, and neither had offspring.

According to the studbook, all individuals with the AMO1 mtDNA haplotype could be traced back to the female founder #7 from Guizhou and all with the COR1 haplotype to the female founder #26 from Fujian (Figure 1A,B). The 2 lineages primarily corresponded to the original Chongqing and Suzhou lines, which were separately managed within the South China tiger population until 1995 (Figure 1A,B). However, the ALT haplotype could not be traced to any female founder recorded in the captive South China tiger population, indicating that the carriers of the Amur tiger mtDNA haplotype might be a result of mismanagement in the breeding program or record inventorying process.

Admixture Detection in the Captive South China Tiger Population

Microsatellite genotypes of the 87 South China tiger samples from 30 loci were combined with data from the 108 voucher tigers and the 5 South China tigers used by Luo et al. (2004) for a Bayesian genetic clustering analysis in STRUCTURE without prior population designation information. The log-likelihood value (Ln probability) of the data set reached the highest when K was 8 (Supplementary Figure S1). Under this scenario, the 108 voucher specimens were grouped by their subspecies affiliation, or, Amur, Indochinese (2 clusters), Malayan, Sumatran, and Bengal tigers (Luo et al. 2004). Out of the 92 South China tigers, including 87 collected in this study and 5 previously (Luo et al. 2004), 74 could be clustered into 2 groups that were distinguishable from all other voucher subspecies and roughly corresponded to the Chongqing and Suzhou lineages with high probability (Figures 1A,B and 2A; Supplementary Table S1). The population genetic assignment results were consistent with zoo records that 2 lines were maintained separately for decades until 1995 when cross breeding occurred to reduce the level of inbreeding (Figure 1A,B). Until genetic profiles of historical South China tigers with wild origin were obtained for a thorough understanding of the genealogy of *P. t. amoyensis*, all captive tigers falling into either Chongqing or Suzhou line are recommended to remain in the studbook and considered "authentic" South China tigers.

The remaining 18 tigers, about 20% of the sampled South China tigers, showed various degrees of genetic introgression from other subspecies, possibly due to mistakes introduced during

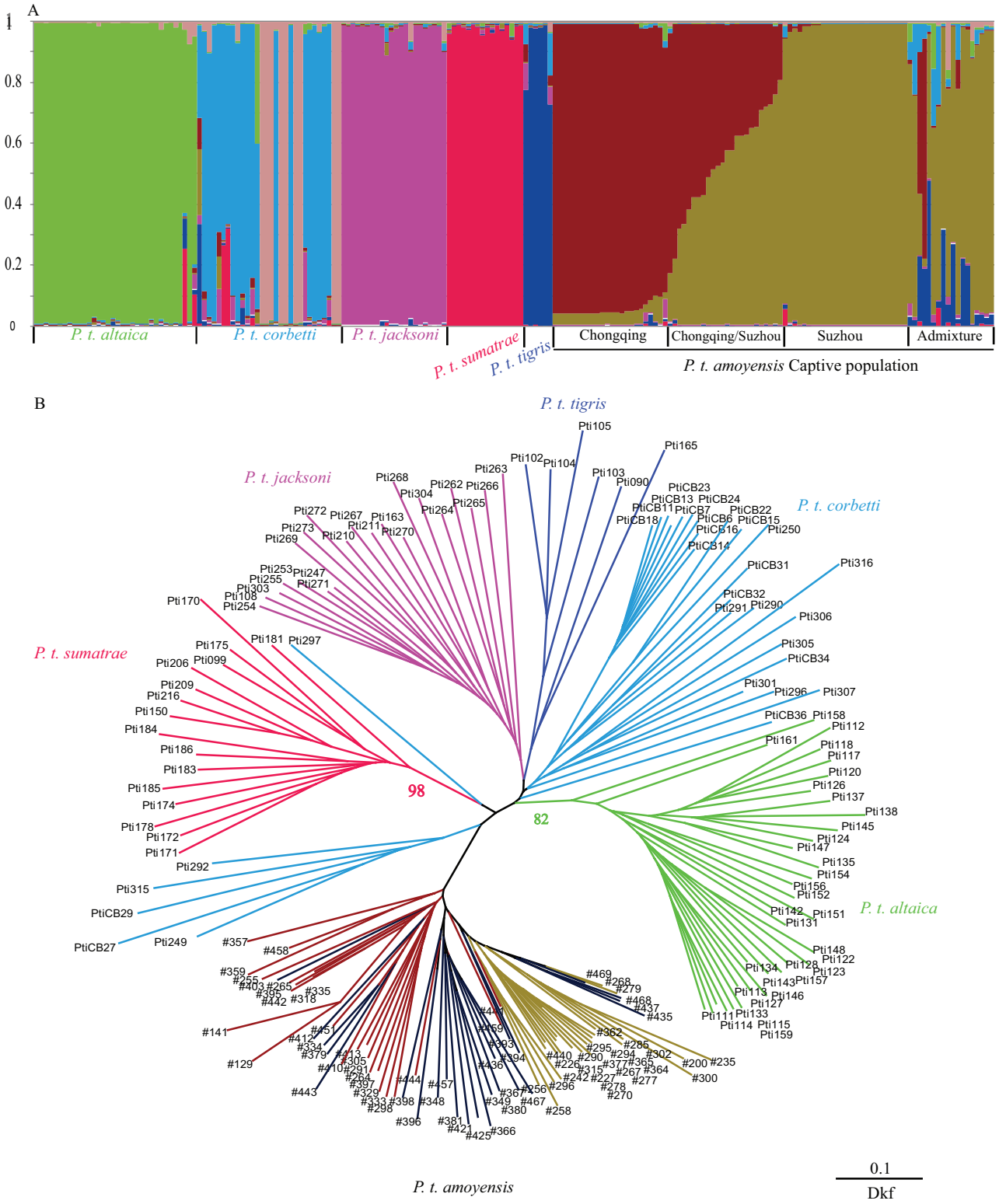

Figure 2. Population genetic structure of captive South China tigers in relation to other tiger subspecies. (**A**) STRUCTURE assignment results of captive South China tigers and voucher subspecies specimens. Here is shown the population genetic structure when $K = 8$ which produced the highest probability among other choices of K (Supplementary Figure S1). The assignment results showed 4 groups in captive South China tigers: Chongqing, Suzhou, the cross-bred with Chongqing and Suzhou (Chongqing/Suzhou), and the admixture with Bengal/Indochinese/Amur tigers (admixture). Each individual was represented by a thin vertical bar, which is partitioned into K colored segments that represent the individual affiliation to each of K clusters. (**B**) The individual-based neighbor-joining (NJ) tree with composite microsatellite genotypes of the 30 microsatellite loci. The NJ trees, based on Dps and Dkf with the (1 – ps/kf) option in MICROSAT, generated similar topologies and only the Dkf tree is shown here. Bootstrap values over 50% are shown on the divergence node. Numbers with # are studbook numbers of the sampled captive South China tigers. Branches of the same color represent tiger individuals of the same subspecies except for South China tigers, in which dark gray represent those identified as the Chongqing line, light gray the Suzhou line, and black the cross breeding between the 2 lines, following the assignment results in STRUCTURE. See online version for full color.

breeding and management (Figures 1A and 2A; Supplementary Table S1). The sources of genetic introgression were identified as being from Bengal, Amur, or Indochinese tigers ($0.05 < q < 0.5$; Figures 1A and 2A; Supplementary Table S1). No genetic admixture from Malayan or Sumatran tiger ($q < 0.07$) was detected within the population. Except for 1 individual (studbook #450, Chongqing line $q = 0.72$), all 18 tigers were identified with admixed genetic ancestry descended from the Suzhou line (0.2–0.99; Figures 1A and 2A; Supplementary Table S1). In particular, among the 14 admixed tigers that shared the Amur tiger ALT mtDNA haplotype, 5 had high nuclear genetic affiliation to Suzhou line ($q > 0.85$) and the remaining 9 contained genetic components from Bengal, Indochinese, or Amur tigers (0.15–0.50) in addition to an assignment to the Suzhou line ($q > 0.45$). For the 4 tigers with COR1 (n = 2) or AMO1 (n = 2) haplotypes, relatively low levels of genetic admixture from Indochinese or Bengal tiger were observed (0.05–0.25; Figures 1A and 2A; Supplementary Table S1).

The postulation that genetic admixture in the 18 tigers was derived from management mistakes is also evident in parentage verification, with mother–offspring mtDNA haplotype mismatches in conjunction with microsatellite genotype discrepancies identified from these individuals (Figure 1A; Supplementary Table S1). As a result, subsequent exclusion of these and their offspring from the captive South China tiger breeding program is recommended. Although additional parentage discordances were detected within the pedigree (Figure 1B), we tentatively conclude that these paternity mistakes probably occurred within the population, rather than accidental admixture from individuals outside the studbook.

Genetic Association of VSA South China Tigers With Other Subspecies

Individual-based NJ trees of all voucher subspecies and captive South China tigers, excluding the 18 individuals with accidental admixture, were constructed based on genetic distance matrices calculated from the proportion of shared alleles (Dps) or kinship coefficient (Dkf). NJ trees based on either Dps or Dkf formed a concordant topology that showed strong genetic differentiation corresponding to major tiger subspecies division (Figure 2B). Consistent with the pattern reported by Luo et al. (2004), Indochinese tigers formed 2 distinct clusters. Although all captive South China tigers sampled in this study formed a separate group, they closely aligned with one minor cluster of the Indochinese tiger consisting of 5 samples (Figure 2B) and could be further divided into 3 subgroups: Chongqing line, Suzhou line, and a cross-bred group of the 2 lines (Figure 2B). The NJ tree is in general agreement with the Bayesian population clustering results.

To further examine the genetic ancestry of present South China tigers in relationship to other tiger subspecies, STRUCTURE analysis was performed with only unrelated tigers to exclude the influence of familial relatedness in the assignment test. According to the pedigree, only one pair of such individuals, #242 (Pti-217 in Luo et al. 2004) and #129 (Pti-219), corresponding to the Chongqing and Suzhou lines, respectively, were available. The highest log-likelihood value of STRUCTURE clustering (Ln probability) was obtained when K was set to 6 (Supplementary Figure S1). Under this scenario, South China tigers were undistinguishable to one lineage of the Indochinese tiger (Supplementary Figure S2). When K was increased to 7 and 8, additional divisions occurred within Indochinese tigers but not between the 2 South China tigers and others (Supplementary Figure S2), which was consistent with the pattern observed in the NJ tree (Figure 2B) and indicated a close genetic association between *P. t. amoyensis* in captivity and *P. t. corbetti*.

Populations' Statistics of Captive South China Tigers

Excluding the 18 tigers verified to have genetic introgression from Bengal, Indochinese, or Amur tigers (Figure 1A; Supplementary Table S1), the remaining 69 individuals representing the captive South China tigers at present (not including the 5 individuals used by Luo et al. 2004) were used to evaluate the genetic relatedness within the captive population. Genetic diversity of the captive South China tiger was compared with captive Amur tigers sampled from European and North American coordinated breeding programs (n = 32; data from Luo et al. 2008), 2 of the world's most successful conservation breeding programs for ex situ tiger conservation (Figure 3; Table3).

Levels of microsatellite genetic diversity in the captive populations of South China tigers and Amur tigers were comparable in terms of the average number of alleles per microsatellite locus (3.7667 vs. 3.8333), mean microsatellite variance (2.0347 vs. 2.1383), and average repeat per locus (4.7333 vs. 4.7000). The captive Amur tigers harbored higher level of microsatellite heterozygosity ($H_O = 0.505$ and $H_E = 0.516$) than South China tigers ($H_O = 0.441$ and $H_E = 0.471$).

The distribution of pairwise genetic relatedness (R_{XY}) values showed that Amur tigers within the coordinated breeding captive programs were more outbred, relative to individuals within the South China tiger captive population (Figure 3). Captive Amur tigers had a mean R_{XY} of 0.07593 ± 0.13647, whereas captive South China tigers had a mean R_{XY} of 0.10257 ± 0.16593 (Table 3). The Mann–Whitney U test demonstrated that captive South China tigers were significantly more related to each other than between individual captive Amur tigers ($P < 0.0001$; Figure 3). This was consistent with the higher inbreeding coefficient within the South China tiger captive population ($F_{IS} = 0.0639$; Table 3) than that in the Amur tiger captive population ($F_{IS} = 0.0332$; Table 3), which affirmed a high level of inbreeding in current captive South China tiger population.

Discussion

We reported a comprehensive genetic assessment of the world's last living South China tiger population now surviving only in

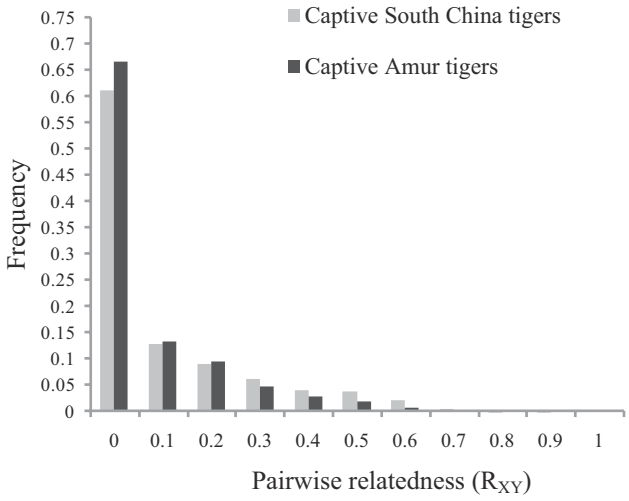

Figure 3. Distribution of pairwise relatedness values for all pairwise comparisons within captive Amur tigers (*P. t. altaica*, $n = 32$) and captive South China tigers (*P. t. amoyensis*, $n = 69$). The data for Amur tigers came from Luo et al. (2008).

Table 3. Genetic diversity in the captive South China tiger and Amur tiger populations

		Amur tigers	South China tigers
Population size (public data)	Wild	450[b]	0[a]
	Captive	421[b]	144
Sample size in this study		32	92[c]
Inbreeding index based Studbook		Unknown	0.3584
Microsatellite loci	Average observed heterozygosity	0.505	0.441
	Average expected heterozygosity	0.516	0.471
	Average number of alleles	3.8333	3.7667
	Average microsatellite variance	2.1383	2.0347
	Average allele size range	4.7000	4.7333
	F_{IS}	0.0332	0.0639
	Average relatedness (R)	0.07593	0.10257
mtDNA haplotype codes (sample size)		ALT (32)	AMO1 (45), COR1 (24)

[a]Data from Tilson et al. (2004).
[b]Data from Luo et al. (2008).
[c]Data including South China tigers used by Luo et al. (2004).

captivity. An explicit tiger subspecies-diagnostic system, including 4 kb of mtDNA sequences and 30 microsatellite loci, was applied to 92 tigers recorded in the studbook for *P. t. amoyensis* or over 75% of the entire captive population at the time of collection. Three mtDNA haplotypes were found in this captive population, including a unique *P. t. amoyensis* lineage (AMO1), a common *P. t. corbetti* lineage (COR1) widespread in Thailand, Vietnam, and Cambodia, and a *P. t. altaica* haplotype (ALT) fixed in Amur tigers from northeast Asia.

Because nuclear microsatellite analysis revealed 2 major clusters, whose matrilineal ancestry can be traced back to only 2 female wild-caught founders from Guizhou (#7) and Fujian (#26), the existence of 3 mtDNA haplotypes in the population implied erroneous introduction of unknown individuals into the studbook. In combination with parentage tests, 18 tigers were verified to be the consequence from such accidental breeding. Genetic admixture from Bengal, Indochinese, and Amur tigers was evident in these individuals, including 14 sharing the ALT haplotype, 2 with COR1, and 2 with AMO1 (Supplementary Table S1; Figures 1A,B and 2A). Due to the various genetic backgrounds of tigers within the captive facilities in China, it is impossible to trace the exact source of such admixture. It is most likely that only a few tigers with admixed genetic background were incidentally introduced and we recommend that these individuals with apparent genetic introgression be excluded from the South China tiger captive breeding program.

Phylogenetic and Bayesian clustering analyses based on composite microsatellite genotypes consistently showed that the present captive South China tiger population is closely associated with Indochinese tigers (Figure 2; Supplementary Figure S2). According to the studbook and pedigree, the widespread Indochinese tiger mtDNA haplotype COR1 found in the captive South China tiger population can be traced back to tigress #26 from Fujian in southeast China. If the geographical origin of #26 and the pedigree record were correct, this pattern suggested a complicated genetic background in *P. t. amoyensis*. Indeed, because the geographical boundary between *P. t. amoyensis* and *P. t. corbetti* is poorly defined, the distribution of mtDNA lineages similar to Indochinese tigers might be natural in southeast China. Intriguingly, Liu et al. (2018) retrieved whole genome data from #129 and detected cytonuclear discordance in its phylogenomic placement, which is basal in the mitogenome phylogeny but closely associated with the Amur tiger clade in the autosomal tree. The key to answer whether this association was due to the uncertainty in the recorded founders' actual sources, or indeed reflected the genetic connectivity between natural populations, would only be available with wild-origin South China tiger specimens from museums or private collections and from a whole genome perspective.

The mean inbreeding coefficient of the present captive South China tiger population as of 2016 was 0.3584 (0.1797–0.4922; Table 3). Without additional founders, the small captive population might eventually suffer from intensified inbreeding. Considering the genetic association between the captive South China tiger and Indochinese tiger populations, it might be an option to introduce additional Indochinese tigers from Southeast Asia into the population to enlarge the gene pool of this captive population. If stringent genetic management could be subsequently ensured and inbreeding depression indeed become profound, genetic intervention is absolutely necessary. A similar strategy has proven successful in rescuing the small, highly inbred wild Florida panther population from severe consequence of genetic depletion (Johnson et al. 2010). However, owing to a lack of understanding of the original genetic composition of *P. t. amoyensis*, irreversible introduction of new Indochinese tigers into the captive South China tiger population may bear the risk of compromising the genetic integrity of *P. t. amoyensis*. At this moment, the question remains whether or not the benefits brought by reduced inbreeding due to translocations would outweigh the tangible threats of the genetic integrity of the world's last living South China tigers.

Although a few tigers with unknown genetic background were accidentally introduced into the captive South China tiger population in China, the remaining captive tigers in the program, after excluding the obviously admixed individuals, still exhibit moderate levels of genetic variability without signs of severe genetic depletion or extreme inbreeding depression. With improved husbandry, the rate of cub mortality has decreased from more than 50% to less than 40%, and a healthier age structure and sex ratio have been established. By November 2016, 144 South China tigers were maintained at zoo facilities across China after excluding all individuals bearing the Amur tiger mtDNA haplotype. Among these 65 tigers

were younger than 4 years old and 73 were male (Yin 2016). The mean number of alleles per microsatellite locus in South China tigers was similar comparable to that observed in small natural populations such as the Florida panther, Asian lion, and Amur leopard (Luo et al. 2004). The overall level of genetic variability, as measured by the mean observed and expected heterozygosity in the captive South China tiger population, was only slightly lower than that in the captive population of Amur tigers in North America, one of the most successful ex situ conservation programs for the tiger (Luo et al. 2008), and similar to the captive Amur leopard population in Russia (Uphyrkina et al. 2002). We recognize that the captive South China tiger population still has some potential for population growth given well-coordinated genetic management strategies. This study provides critical information that is fundamental to both the understanding of the current status of South China tigers and their future management and conservation.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Conflict of Interest

The authors declare that no conflict of interest exists.

Data Availability

All data uploaded as online Supplementary Material.

References

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Notes*. 10:564–567.

Fàbregas MC, Fosgate GT, Koehler GM. 2015. Hunting performance of captive-born South China tigers (*Panthera tigris amoyensis*) on free-ranging prey and implications for their reintroduction. *Biol Conserv*. 192:57–64.

Felsenstein J. 1989. PHYLIP – phylogeny inference package (version 3.2). *Cladistics*. 5:164–166.

Goodrich J, Lynam A, Miquelle D, Wibisono H, Kawanishi K, Pattanavibool A, Htun S, Tempa T, Karki J, Jhala YV, *et al*. 2015. *Panthera tigris*. The IUCN Red List of Threatened Species 2015: e.T15955A50659951. doi:10.2305/IUCN.UK.2015-2.RLTS.T15955A50659951.en

Hilzheimer M. 1905. Über einige Tigerschädel aus der Strabburger zoologischen Sammlung. *Zool Anz*. 28:594–599.

Johnson WE, Onorato DP, Roelke ME, Land ED, Cunningham M, Belden RC, Mcbride R, Jansen D, Lotz M, Shindle DB, *et al*. 2010. Genetic restoration of the Florida panther. *Science*. 329:1641–1645.

Kalinowski ST, Wagner AP, Taper ML. 2006. ML-Relate: a computer program for maximum likelihood estimation of relatedness and relationship. *Mol Ecol Notes*. 6:576–579.

Kitchener AC. 1999. Tiger distribution, phenotypic variation and conservation issues. In: Seidensticker J, Christie S, Jackson P, editors. *Riding the tiger: tiger conservation in human-dominated landscapes*. Cambridge (UK): Cambridge University Press. p. 19–39.

Kitchener AC, Breitenmoser-Würsten Ch, Eizirik E, Gentry A, Werdelin L, Wilting A, Yamaguch N, Abramov AV, Christiansen P, Driscoll CA, *et al*. 2017. *A revised taxonomy of the Felidae. The final report of the Cat Classification Task Force of the IUCN/SSC Cat Specialist Group*. Cat News 11:1–79.

Kitchener AC, Yamaguchi N. 2010. What is a tiger? Biogeography, morphology, and taxonomy. In: Tilson RL, Nyhus PJ, editors. *Tigers of the world, the science, politics and conservation of Panthera tigris*. 2nd ed. New York: Elsevier/Academic Press. p. 53–84.

Liu YC, Sun X, Driscoll C, Miquelle DG, Xu X, Martelli P, Uphyrkina O, Smith JLD, O'Brien SJ, Luo SJ. 2018. Genome-wide evolutionary analysis of natural history and adaptation in the world's tigers. *Curr Biol*. 28(23):3840–3849.

Liu ZH, Yuan YC. 1983. The resources of the South China tiger in China. *Wildlife (China)*. 4:20–22.

Lu H, Sheng H. 1986. Distribution and status of the Chinese tiger. In: Miller S, Everett D, editors. *Cats of the world: biology, conservation and management*. Washington (DC): National Wildlife Federation. p. 51–58.

Luo SJ, Johnson WE, Martenson J, Antunes A, Martelli P, Uphyrkina O, Traylor-Holzer K, Smith JLD, O'Brien SJ. 2008. Subspecies genetic assignments of worldwide captive tigers increase conservation value of captive populations. *Curr Biol*. 18:592–596.

Luo SJ, Johnson WE, O'Brien SJ. 2010a. Applying molecular genetic tools to tiger conservation. *Integ Zool*. 5:351–362.

Luo SJ, Johnson WE, Smith JLD, O'Brien SJ. 2010b. What is a tiger? Genetics and phylogeography. In: Tilson RL, Nyhus PJ, editors. *Tigers of the world, the science, politics and conservation of Panthera tigris*. 2nd ed. New York: Elsevier/Academic Press. p. 35–51.

Luo SJ, Kim JH, Johnson WE, Miquelle DG, Huang SQ, Pan WS, Smith JLD, O'Brien SJ. 2006. Proceeding in phylogeography and genetic ancestry of tigers (*Panthera tigris*) in China and across their range. *Zool Res (China)* 27(4):441–448.

Luo SJ, Kim JH, Johnson WE, Walt JVD, Martenson J, Yuhki N, Miquelle DG, Uphyrkina O, Goodrich JM, Quigley HB. 2004. Phylogeography and genetic ancestry of tigers (*Panthera tigris*). *PLoS Biol*. 2(12):2275–2293.

Minch E, Ruiz-Linares A, Goldstein DB. 1995. MICROSAT. Available from: http://hpgl.stanford.edu/projects/microsat/ (accessed 14 October 2006).

Nyhus P. 2008. *Panthera tigris ssp. amoyensis*. The IUCN Red List of Threatened Species 2008: e.T15965A5334628. doi:10.2305/IUCN.UK.2008.RLTS.T15965A5334628.en.

Pritchard JK, Stephens M, Donelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics*. 155:945–959.

Qin Y, Nyhus PJ, Larson CL, Carroll CJW, Muntifering J, Dahmer TD, Jun LH, Tilson R. 2015. An assessment of South China tiger reintroduction potential in Hupingshan and Houhe National Nature Reserves, China. *Biol Conserv*. 182:72–86.

Sambrook J, Fritsch E, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. New York: Cold Spring Harbor Laboratory Press.

Tan B. 1987. Status and problems of captive tigers in China. In: Tilson R, Seal U, editors. *Tigers of the world: the biology, biopolitics, management, and conservation of an endangered species*. Park Ridge (NJ): Noyes Publications. p. 134–148.

Tilson R, Defu H, Muntifering J, Nyhus PJ. 2004. Dramatic decline of wild South China tigers *Panthera tigris amoyensis*: field survey of priority tiger reserves. *Oryx*. 38(1):40–47.

Uphyrkina O, Miqudlle D, Quigley H, Driscoll C, O'Brien SJ. 2002. Conservation genetics of the Far Eastern leopard (*Panthera pardus orientalis*). *J Hered*. 93:303–311.

Van Oosterhout C, Hutchinson B, Wills D, Shipley P. 2004. Microchecker: software for identifying and correcting genotyping errors in microsatellite data. *Mol Ecol Notes*. 4:535–538.

Wei K, Zhang Z-H, Zhang W-P, Shen F-J, He G-X, Hou R, Xie Z, Liu NL, Yue BS. 2005. Sequence variation of mitochondrial DNA ND5 in captive South China tigers (*Panthera tigris amoyensis*). *J Nat Hist*. 39(46):3979–3984.

Wilting A, Courtiol A, Christiansen P, Niedballa J, Scharf AK, Orlando L, Balkenhol N, Hofer H, Kramer-Schadt S, Fickel J, *et al*. 2015. Planning tiger recovery: understanding intraspecific variation for effective conservation. *Sci Adv*. 1(5:e1400175.

Xu YC, Fang SG, Li ZK. 2007. Sustainability of the South China tiger: implications of inbreeding depression and introgression. *Conserv Genet*. 8(5):1199–1207.

Xue H-R, Yamaguchi N, Driscoll CA, Han Y, Bargal GK, Zhuang Y, Mazak JH, Macdonald DW, O'Brien SJ, Luo S-J. 2015. Genetic ancestry of the extinct Javan and Bali tigers. *J Hered*. 106(3):247–257.

Yin YZ. 2016. *Studbook of the South China tiger*. (China): Chongqing: Chongqing Zoological Garden.

Zhang W, Yue B, Wang X, Zhang X, Xie Z, Liu N, Fu W, Yuan Y, Chen D, Fu D, *et al*. 2011. Analysis of variable sites between two complete South China tiger (*Panthera tigris amoyensis*) mitochondrial genomes. *Mol Biol Rep*. 38:4257–4264.