Sequence variation of mitochondrial DNA ND5 in captive South China tigers (*Panthera tigris amoyensis*)

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(Accepted 5 January 2006)

Abstract
This paper represents the first study of the genetic diversity of captive South China tigers *Panthera tigris amoyensis* by sequence variation in a fragment of mitochondrial DNA (mtDNA) NADH dehydrogenase subunit 5 (ND5) gene. Two haplotypes (haplotype I and haplotype II) were identified and the captive population had a bias towards haplotype I. This subspecies as a whole demonstrated an extremely low level of genetic diversity with the nucleotide diversity of 0.057 ± 0.021%. The mtDNA ND5 variations detected in the present study could provide significant information to the studbook data of the South China tiger.

Keywords: Captive population, genetic diversity, haplotype, ND5, Panthera tigris amoyensis

Introduction
Among the five extant subspecies of tiger in the world, the South China tiger *Panthera tigris amoyensis* is the only one endemic to China. It is classified as “Critically Endangered” in the 2004 International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species, and listed in Appendix I of the Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES). Historically, the distribution range of this animal stretched over 2000 km from east to west and 1500 km from north to south in China. Unfortunately, uncontrolled hunting and extensive deforestation resulted in a precipitous decline in the wild population and it was evaluated to be extinct in the wild in 2004 based on the results of the Sino-American field survey project (Tilson et al. 2004).
According to the South China tiger studbook (Yin 2003) established by the Chinese Association of Zoological Gardens (CAZG), the first captive breeding of the South China tiger started in 1955 when a female individual was captured from the wild in Sichuan province, and the first successful breeding was achieved in Qianling Park of Guizhou Province in 1963. By October 2003, there were 64 living individuals in captivity and they all were derived from six wild-captured founders (two males and four females). Although the size of captive population grew gradually after 1973, the reduced reproductive fitness implicated high levels of inbreeding and low genetic diversity (Chen et al. 2001). A gene drop analysis indicated that the gene diversity of the captive population had decreased while the inbreeding coefficient had increased since 1977 (Wang et al. 2003). Further, uneven sex ratio and fecundity declines may result in a rapid loss of genetic diversity of the maternal lineage (Tilson et al. 1997). All this evidence shows that the current breeding status of captive South China tigers may threaten the long-term survival of this subspecies.

Mitochondrial DNA (mtDNA) has now been widely applied in population genetics and conservation biology of endangered animals because of its high mutation rate. In exhibiting relatively high rates of mutation in the Felidae (Lopez et al. 1997), the NADH dehydrogenase subunit 5 (ND5) seems to be the most appropriate gene for resolving the phylogenetic relationships and genetic diversity analysis within a feline genus in which genetic diversification is supposed to be low. Therefore, for the first time, we examined the genetic diversity of the captive South China tiger population by means of a detailed analysis of the mtDNA ND5 sequences.

**Materials and methods**

*DNA extraction, amplification, and sequencing*

Blood and hair samples from a total of 46 tiger individuals were collected in 2004 from 11 zoos (see Table I). Total genomic DNA was isolated and purified by proteinase K digestion and phenol–chloroform extraction following standard protocols (Sambrook et al. 1989). A portion of the mtDNA ND5 gene sequence believed to be highly polymorphic within the Felidae (Johnson and O’Brien 1997) was amplified. The slightly modified primers ND5-F, 5’-TGCAACTCCAAATAAAAAG-3’, and ND5-R, 5’-GCATGTACCACATTGAGA-3’ (Johnson et al. 1998) were used for both PCR and sequencing. The PCR amplification was performed using 50 ng of template DNA, 5 μl of 10× reaction buffer, 1.5 mM MgCl₂,

| Sample sources       | Individuals sampled | Blood samples | Hair samples |
|----------------------|---------------------|--------------|-------------|
| Chongqing Zoo        | 3                   | 3            |             |
| Guangzhou Zoo        | 6                   | 2            | 4           |
| Fuzhou Zoo           | 4                   | 4            |             |
| Xiamen Zoo           | 1                   | 1            |             |
| Nanchang Zoo         | 2                   | 1            | 1           |
| Jiujiang Zoo         | 1                   | 1            |             |
| Wuhan Zoo            | 1                   | 1            |             |
| Changsha Zoo         | 1                   | 1            |             |
| Luoyang Zoo          | 3                   | 2            | 1           |
| Shanghai Zoo         | 16                  | 10           | 6           |
| Suzhou Zoo           | 8                   | 8            |             |
| Total                | 46                  | 24           | 22          |
200 μM each dNTP, 1 μM each primer and 1.5 units of Taq DNA polymerase under the following conditions: denaturation for 5 min at 95°C; amplification consisting of 35 cycles of 40 s at 95°C, 50 s at 42°C, and 55 s at 72°C; followed by an extension of 10 min at 72°C. Direct sequencing of the PCR product was performed in both directions using an ABI 377 automatic sequencer.

Data analysis

Sequences were aligned using the ClustalX 1.8 program (Thompson et al. 1997). Initial sequence comparisons and measures of variation were performed using MEGA version 2.1 (Kumar et al. 2001). The nucleotide diversity (\(\pi\)) of the sequences, defined as the average number of pairwise nucleotide changes per site (Nei and Li 1979), was calculated according to Nei (1987) using the program DnaSP 3.14 (Rozas and Rozas 1999). Also the neutrality tests, Tajima’s D (Tajima 1989), were conducted using this software.

Results

The fragments of 345 bp of the mitochondrial ND5 gene were amplified and sequenced in 46 South China tiger individuals. By comparing our sequences with the reported feline mitochondrial complete sequences and a Numt sequence (GenBank accession no. U20753, U20754; Lopez et al. 1996), it was unlikely that we had sequenced a nuclear pseudogene which had been believed to be a potential problem for variability estimations in tigers (Cracraft et al. 1998; Luo et al. 2004). Among these 46 sequences, the only variable site (0.29% of the entire sequence) was a transition at position 172, where “T” appeared in 41 individuals (defined as haplotype I) compared to five tigers of “C” (haplotype II). This transition (T→C) resulted in an amino acid substitution (Val→Ala). For the dominant haplotype I, mean base-pair composition at all three positions was 33.6% A, 31.6% T, 26.3% C, and 8.5% G, resembling biases against guanine in portions of the mitochondria described in the domestic cat (Lopez et al. 1997) and other non-felid species (Ikemura 1985; Sharp et al. 1988). The nucleotide diversity of the total individuals was only 0.057 ± 0.021% (see Table II). A negative value of Tajima’s D (−0.17787) was obtained, and was found to be not significantly different from zero (\(P > 0.10\)).

Discussion

The captive South China tigers demonstrated an extremely low level of genetic diversity with a nucleotide diversity of 0.057 ± 0.021%. Besides, a significant bias towards haplotype I was found in this captive population. The disproportionate haplotype distribution is presumably linked to the haplotype composition of the founders, the different individual

| Population | \(N\) | \(N_{hap}\) | \(N_{II}\) | \(S\) | \(\pi\) (%) | Tajima’s D |
|------------|------|--------|--------|-----|---------|-----------|
| Captive    | 46   | 2      | 41     | 5   | 1       | −0.17787* |

\(N\), number of individuals; \(N_{hap}\), number of haplotypes; \(N_{II}\), number of haplotype I; \(N_{II}\), number of haplotype II; \(S\), number of variable sites; \(\pi\), nucleotide diversity. *Estimated using Kimura two-parameter distance (Kimura 1980). *\(P > 0.10\), not significant.
reproductive capability, and the human choices in focusing on a few individuals possessing good reproductive ability.

Extremely low levels of genetic diversity are commonly found in large felid species, including African cheetah (O’Brien et al. 1983), lions of the Ngorongoro crater (Packer et al. 1991), Florida panther (Roelke et al. 1993), and Amur tigers (Russello et al. 2004). A similar situation has also been reported in a wide variety of taxa including black rhinoceros (Moehlman et al. 1996), whooping crane (Glenn et al. 1999), North American wolverine (Kyle and Strobeck 2001), and Aldabra tortoise (Palkovacs et al. 2003). These studies revealed that the presence of the founder events or bottleneck effects in recent or historic times resulted in genetic impoverishment.

Tajima’s D statistic is widely used to examine the distribution of DNA polymorphisms within species. It is based on the examination of the relationship between the estimates of genetic variation which can be calculated from the number of segregating sites and the nucleotide diversity (Tajima 1989). The presence of deleterious mutations or a recent founder event/bottleneck is generally expected to cause negative values of Tajima’s D (Hudson 1996; Nielsen 2001), which may imply that due to the recent founder effect and the subsequent population building up, the current South China tiger population does not reach the equilibrium and therefore Tajima’s D is expected to be found negative (D = \(-0.17787\)).

A long-term goal for the captive breeding of many endangered species is to eventually reintroduce individuals into the wild. For South China tigers, we must not lose sight of the fact that the wild population may already be functionally extinct, and therefore the remaining captive tigers are recognized as being the only back-up for the recovery of the wild population. Despite the success of captive breeding and the increase in individual numbers under intensive management, the extremely low genetic diversity is still the greatest menace to South China tigers, which is likely to make it more prone to extinction from new disease or other environmental changes. Therefore, genetic diversity in the South China tiger captive population is a major concern in the current conservation of this rare subspecies. The variation of ND5 sequences could provide significant information for the studbook for this subspecies. Since the distribution of haplotypes in this captive population was biased towards haplotype I, special attention should be paid to individuals with haplotype II when considering captive breeding management, so that haplotype II might not be lost by chance and offspring with a better haplotype distribution could be produced. Together with the help of pedigree information, it may be possible to minimize genetic loss by choosing individuals with the lowest relationship in the population to be parents of the subsequent generation. This would result in the highest degree of retention of genetic variation.

Acknowledgements

We are grateful to Jun Chen and Yuzhong Yin from Chongqing Zoo; Cuilian Huang, Qinhui Cai, and Houneng He from Guangzhou Zoo; Yuanzhi Chen and Yao Tang from Fuzhou Zoo; Yueying Liu from Nanchang Zoo; Qingtao Lu from Changsha Zoo; Xinhui Sun, Yu’an Zong, and Xingzuo Wang from Luoyang Zoo; Enle Pei and Yaohua Yuan from Shanghai Zoo; Gongqing Huang and Daqing Liu from Suzhou Zoo; and Wuzhong Zheng from Xiamen Zoo, for providing the samples used in this study. For financial support we acknowledge the Research Fund for Giant Panda Breeding of Chengdu, the North of England Zoological Society, and the Chinese Association of Zoological Gardens (CAZG).
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