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Use of oligonucleotide fingerprinting and faecal DNA in identifying the distribution of the Chinese tiger (*Panthera tigris amoyensis* Hilzheimer)

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Abstract. Chinese tiger (*Panthera tigris amoyensis* Hilzheimer) was once distributed in the Zhejiang Province, China, but there have not been any tiger sightings in this province since 1985. Fortunately, within the boundaries of the Fengyangshan-baishanzu State Natural Reserve, gamekeepers have found 'tiger footprints' and 'tiger faeces' many times since 1998. However, these tracks may have been left by the leopard or clouded leopard, which leave similar tracks. The presence of the Chinese tiger in Zhejiang Province was demonstrated using DNA fingerprinting and faecal DNA. The study not only revealed a new habitat for Chinese tigers, Zhejiang Province, but also provided an effective scatology method to identify the distribution of tigers and other wild felines.

Introduction

The Chinese tiger (*Panthera tigris amoyensis* Hilzheimer), a South China tiger, is an endangered species endemic to China, which is listed in Category I of the State Key Protected Wildlife List and in the EN Category of the IUCN, and presented in Appendix I of CITES (Zhuge 1990; Wang 1998). One hundred years ago, Chinese tigers were widely distributed in the South of China, but large-scale habitat loss, especially in the late 1950s and early 1960s, led to rapid reduction of populations. Today the estimated population comprises only 20–30 individuals (Wang 1998). Before 1985, there were occurrences of tiger sightings, tiger faeces, tiger scratches, tiger roaring and cases of tigers preying on livestock in Zhejiang Province. However, no tracks have been observed since 1985 and researchers have argued that the Chinese tiger has become extinct in Zhejiang Province and that species distribution has shrunk to the four provinces Fujian, Hunan, Guangdong and Jiangxi (Nowell and Jackson 1996; Wang 1998; Jing 1999). Their wide range and great

Table 1. Details relating to the samples.

Common name	Species/subspecies	Number of different samples (M, F)		
		Skin	Faeces	Hair
Unknown	Unknown	–	31	–
South China tiger	*Panthera t. amoyensis*	3♂, 3♀	1♂, 1♀	–
Amur tiger	*Panthera t. altaica*	4♂, 3♀	–	1♂, 1♀
Bengal tiger	*Panthera t. tigris*	2♂, 2♀	–	–
Leopard	*Pantera p. fuscus*	4♂, 2♀	–	2♂, 3♀
Clouded leopard	*Neofelis nebulosa*	3♂, 3♀	–	3♂, 3♀
Muping tufted deer	*Elaphudus c. michianus*	3♂, 3♀	–	2♂, 2♀
Chinese water deer	*Hydropote inermis*	2♂, 3♀	–	3♂, 3♀
Chinese muntjac	*Muntiacus r. reevesi*	4♂, 4♀	–	3♂, 3♀
Black muntjac	*Muntiacus crinifrons*	3♂, 3♀	–	2♂, 2♀

mobility make Chinese tigers extremely difficult to detect, and the state government and researchers have not obtained direct evidence of their distribution in some areas for roughly 20 years. As a result, the existing distribution and the number of wild populations need further confirmation.

Fengyangshan-baishanzu State Natural Reserve lies in Qingyuan County of Zhejiang Province. Since October 1998, gamekeepers in this reserve have found many signs such as ‘tiger footprints’, ‘tiger faeces’ and so on. After analyzing traditional morphology parameters such as faecal size and food type, we estimated that the tracks may be evidence of Chinese tiger presence, but it is also possible that the traces were left by the leopard or clouded leopard in this region. To confirm the existence of the Chinese tiger in Zhejiang Province, we conducted an ecological investigation in the province, and performed molecular identification.

Materials and methods

Materials

From October 1, 1998 to February 18, 1999, the investigation group collected ‘tiger faeces’ in Fengyangshan-baishanzu State Natural Reserve and obtained 44 fresh or comparatively fresh faecal samples. Each faecal sample was dried over the flame of hot coals in a brazier until completely dry. Subsequently, each scat sample was stored in a plastic bag with silica bead packets at the bottom to desiccate the scat and halt microbial activity, and was labeled. Since many workers in the Reserve could not recognise ‘tiger faeces’, some faeces from other species were wrongly sent to our laboratory. We selected 31 faeces, most probably from real tigers, for identification according to the morphological characteristics of tiger faeces. Additionally, two faecal samples of one male and one female were collected as standard samples from

University, the Specimen Museum of Sichuan University, the Tibet Specimen Museum, and the Specimen Museum at the Institute of Zoology at the Chinese Academy of Science provided us with 54 dried skin samples. Hangzhou Zoo and Chengdu Zoo supplied 33 hair samples. These control samples were from different subspecies of tiger (*Panthera tigris altaica* Temminck, *P. t. tigris* Linnaeus and *P. t. amoyensis* Hilzheimer), leopard (*Panthera pardus fusca* Meyer), clouded leopard (*Neofelis nebulosa nebulosa* Griffith), Chinese water deer (*Hydropotes inermis inermis* Swinhoe), Chinese muntjac (*Muntiacus reeves reeves* Ogilby), black muntjac (*Muntiacus crinifrons* Sclater) and muping tufted deer (*Elaphudus cephalophus michianus* Swinhoe) (Table 1). The samples of each species or subspecies were collected from two to four wild populations and one to two captive populations.

Isolation of DNA

Dried faecal material (7 g) was soaked in 300 ml of TNE buffer (10 mM Tris–HCl, 100 mM NaCl, 10 mM EDTA, pH 7.8) and agitated until the faeces were completely dispersed. The remains of bones and skins from tiger prey were removed and other faecal sediments were transferred to a 50 ml tube with TNE buffer, which was centrifuged at 1500–2000 $\times$ *g* for 5 min. The top aqueous layer was pipetted to a clean tube and centrifuged at 4000 g for 10 min. The sediments were collected and resuspended in TNE buffer containing 2% sodium dodecyl sulfate (SDS) and 100 μg/ml of proteinase K. The extraction mixture was incubated for 4 h at 55 °C, and extracted twice with an equal volume of phenol, followed by another extraction with an equal volume of chloroform. The DNA was precipitated in a 150 ml cup by the addition of two volumes of 95% ethanol and formed a stringy gelatinous clump by gently stirring. The stringy DNA was pipetted to a 1.5 ml tube, and dissolved in a small volume of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) after drying by a vacuum pump. DNA was extracted from dried skins according to Fang and Wan (2002), while isolation of hair DNA was performed as described by Fang et al. (2002a).

Probe preparation

An oligonucleotide probe (5′-TCC TCA GCC CAC TCC ACC TCA GCC GGG-3′, pta_1) was synthesized on an ABI-394 DNA synthesizer. The probe was 5′-end labelled with [γ-^{32}P] ATP using T4 polynucleotide kinase according to the protocol of Schäer et al. (1988).

Southern blot analysis

Approximately 5 μg of DNA from each sample was digested with 15 U of a *Hae*III restriction enzyme for over 3 h in 30 μl total volume. We produced an experimental control by mixing DNA fragments from seven standard species including the Chinese tiger, the leopard, the clouded leopard, the Chinese water deer, the Chinese

muntjac, the black muntjac and the muping tufted deer. All DNA fragments were separated by electrophoresis on 0.8% agarose gels for 36 h at 1.5 V/cm. For DNA fingerprinting the gel was dried on a vacuum gel dryer (Bio-rad, FRG).

Prehybridization and hybridization were conducted according to a method described by Bruford et al. (1992) using a 0.25 M phosphate buffer containing 1 mM EDTA, 7% SDS, and 1% BSA as prehybridization and hybridization solutions. Autoradiography was carried out at −80 °C using Kodak XAR 5 X-ray film for periods of 1–4 days. An intensifying screen was used as appropriate.

Results

We successfully extracted DNAs from two known faeces, 33 hair and 54 dried skin samples from different subspecies of tiger, leopard, clouded leopard, Chinese water deer, Chinese muntjac, black muntjac and muping tufted deer, and 31 unknown faecal samples. After all DNA fragments were hybridized with probe pta_1, the DNA banding patterns show that even though every control species/subspecies had several males and several females from different populations, in each Felidae species/subspecies, males produced the same DNA patterns as females but no bands



Figure 1. Comparison of hybridization banding patterns from known and unknown samples. 1. Leopard; 2. Chinese muntjac; 3. Clouded leopard; 4. Black muntjac; 5. Amur tiger; 6. Bengal tiger; 7. Chinese water deer; 8. Mixed DNAs of seven species (i.e. lanes 1–4, 7, 9 and 12); 9. Muping tufted deer; 10. Unknown faecal sample; 11. Standard faeces of Chinese tiger; 12. Standard skin of Chinese tiger; 13. Standard faeces of Chinese tiger; 14. Unknown faecal sample; 15. Muping tufted deer; 16. Mixed DNAs of seven species (i.e. lanes 12, 15, 17 and 20–23); 17. Chinese water deer; 18. Bengal tiger; 19. Amur tiger; 20. Black muntjac; 21. Clouded leopard; 22. Chinese muntjac; 23. Leopard.

Table 2.

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