

The screening of microsatellite DNA loci for the identification of individual Tibetan foxes, using copro-DNA samples

YANG Yingyuan¹, LIU Nan¹, ZUO Qingqiu¹, RENQING Pengcuo³, XIE Fei³, YANG Gang¹, WANG Zhenghuan^{1,2*}

(1 School of Life Sciences, East China Normal University, Shanghai 200062, China)

(2 Shanghai Key Laboratory of Urbanization and Ecological Restoration, Shanghai 200062, China)

(3 Center of Disease Control of Shiqu County, Ganzi, Sichuan Province 627350, China)

Abstract: The Tibetan fox (*Vulpes ferrilata*) has been identified as the main wildlife host of *Echinococcus multilocularis* and *E. shiquicus* in the eastern Tibetan plateau in China. Echinococcosis is a lethal parasitic zoonosis caused by *Echinococcus* spp. endemic to the pastures of the eastern Tibetan plateau. Thus, *Echinococcus* prevalence in Tibetan fox populations is of interest for studies into this disease. Consequently, there is practical significance to evaluate the population size of Tibetan foxes. Therefore we developed noninvasive microsatellite DNA identity analysis techniques using Tibetan fox feces. A total of 48 microsatellite loci were tested for effectiveness, among which 11 were selected to analyze identities of 128 qualified Tibetan fox fecal samples collected in field during July–August, 2011 and 2012 (i. e., 68 in 2011, and 60 in 2012). The number of genotypes (N), expected heterozygosity (H_e), observed heterozygosity (H_o), polymorphism information content (PIC), and probability of identity (PI) were calculated by allelic frequency. N ranged from 4 to 7, H_e was 0.66–0.80, H_o was 0.17–0.68, and PIC was 0.5496–0.7623. The overall copro-DNA PI values of the 11 loci were low ($PI_{biased} = 1.283 \times 10^{-11}$; $PI_{sibs} = 7.572 \times 10^{-5}$). However, the amplification success rate of each microsatellite locus was quite different ranging from 0.926 to 0.176. We then sorted the loci according to their amplification success rates from the highest to the lowest and found PI values of the first six loci with amplification success rates above 60% (i. e., P03, CXX172, CPH6, CPH8, P01i, P08) were already low enough ($PI_{biased} = 2.775 \times 10^{-7}$; $PI_{sibs} = 3.606 \times 10^{-3}$) for individual identification. Therefore the individual identification standards were devised as follows: (1) only copro-DNA samples with successful amplification from at least the first six microsatellite loci were used for further analysis; (2) when all alleles were identical between two samples, the samples were considered to originate from the same individual; (3) in a conservative approach, if just one allele mismatch was observed between two samples, they were also judged to originate from the same individual. We then identified 30 fox individuals from fecal samples in 2011, and 21 individuals from fecal samples in 2012.

Key words: Fecal DNA; Individual identification; Microsatellite DNA; Tibetan fox

基于粪便 DNA 的藏狐微卫星位点筛选及个体识别

杨应远¹ 刘楠¹ 左清秋¹ 仁青彭措³ 谢飞³ 杨刚¹ 王正寰^{1,2*}

(1 华东师范大学生命科学学院, 上海 200062)

(2 上海市城市化过程与生态恢复重点实验室, 上海 200062)

(3 四川省甘孜州石渠县疾控中心, 甘孜 627350)

摘要: 藏狐是我国青藏高原东部多房棘球绦虫和石渠棘球绦虫最主要的野生动物终末宿主。棘球绦虫会导致一类称为棘球绦虫病的致死性人兽共患疾病, 青藏高原东部牧区是该病重要的流行区。因此作为终末宿主, 评估藏狐种群的棘球绦虫感染率对于该病的流行病学研究意义明显。而要获取这方面信息, 首先必须了解藏狐的种群数量。为此, 我们基于非损伤取样的原则, 使用藏狐新鲜粪便作为研究材料, 从已发布的藏狐及近缘种的 48 个微卫星位点中筛选了 11 个用于藏狐粪便 DNA 多态性分析。对 2011–2012 年 7–8 月间收集的 128 份有效藏狐粪便样品 (2011 年 68 份, 2012 年 60 份) 进行特异性 PCR 扩增, 并用琼脂糖凝胶电泳和荧光引物标记法进行基因分型, 根据各位点的等位基因频率计算出各位点的基因型数 (N), 期望杂合度 (H_e)、观测杂合度 (H_o)、多态信息含量 (PIC) 以及不同个体基因型相同概率值 (PI)。结果发现, 各位点 N 介于 4–7, H_e 为 0.66–0.80, H_o 为 0.17–0.68, PIC 为 0.5496–0.7623。11 个位点的累积 PI 值满足个体识别的需要 ($PI_{biased} = 1.283 \times 10^{-11}$;

Foundation items: Natural Science Foundation of China (31071944), and the Shanghai Rising-Star Program (10QA1402200)

Biography: Yingyuan Yang (1988–), master's degree candidate, majored in molecular ecology.

Received date: 2013–11–26; **Accepted date:** 2014–03–04

* Corresponding author, E-mail: zhwang@bio.ecnu.edu.cn.

$PI_{sibs} = 7.572 \times 10^{-5}$)。但是, 由于粪便 DNA 质量差异较大, 不同位点的扩增成功率差异较大 (0.176 – 0.926)。我们发现, 按照扩增成功率由高到低排列, 前 6 个微卫星位点 (P03, CXX172, CPH6, CPH8, P01i, P08) 的扩增成功率均超过 0.6, 且累积 PI 值小于 0.004 ($PI_{biased} = 2.775 \times 10^{-7}$; $PI_{sibs} = 3.606 \times 10^{-3}$), 表明这 6 个位点可以对藏狐进行个体识别。因此, 针对本研究的数据, 制定了如下的个体识别原则: (1) 只有粪便 DNA 至少成功扩增出前 6 个微卫星位点的样品可以进入下一步分析; (2) 所有位点的信息均相同的两个样品被认为是来自同一个体; (3) 保险起见, 如果仅有一对位点信息不相等, 此两个样品依然被判定来自同一个体。在此基础上, 我们从 2011 年样品中识别出 30 个藏狐个体, 从 2012 年样品中识别出 21 个个体。

关键词: 藏狐; 微卫星 DNA; 个体识别; 粪便 DNA

中图分类号: Q346.5

文献标识码: A

文章编号: 1000-1050 (2014) 04-0387-06

1 Introduction

A lethal zoonosis, echinococcosis, has been recognized as being endemic to the pastures of the eastern Tibetan plateau in China (Li *et al.*, 2005). Echinococcosis is caused by canid tapeworms, which belong to the *Echinococcus* genus (Eckert *et al.*, 2001). The Tibetan fox (*Vulpes ferrilata*) has been identified as the main wildlife host of *Echinococcus multilocularis* and *E. shiquicus* in this region (Qiu *et al.*, 1995). Thus, *Echinococcus* prevalence in Tibetan fox populations is of interest for studies into this disease. Consequently, there is practical significance to studying the population size of Tibetan foxes. As a first step to evaluate the population size, we used noninvasive microsatellite DNA analysis to identify Tibetan fox individuals. Fecal samples were used as the source of DNA (copro-DNA) for the analysis (Jiang *et al.*, 2012). Microsatellite DNA was chosen as it has been widely used in demographic studies of wildlife (Harrison *et al.*, 2002; Zhan *et al.*, 2006). However, fecal microsatellite DNA has never been rigorously studied in the Tibetan fox.

In order to develop a practical DNA analysis method for fecal samples, we previously identified 15 microsatellite loci from 36 tissue samples of the Tibetan fox (Li *et al.*, 2011). However, two thirds of these loci could not be amplified successfully or repeatedly from copro-DNA samples. Therefore, to collect enough of microsatellite loci data for copro-DNA individual identification, we investigated a total of 48 published microsatellite loci in the Tibetan fox and other canid species, and 11 loci, including five published by us earlier, were selected. The ability of these loci to distinguish between individuals was tested on fecal samples that were used to evaluate numbers of Tibetan foxes.

2 Materials and methods

Field studies were performed in area of an approx-

imately 100 km² of Yunbo Gou, Shiqu County, Sichuan Province, China (32°19' – 34°20'N, 97°20' – 99°15'E). The area is a wide valley that is dominated by alpine meadow, with an elevation of 4 300 m above sea level. A small river runs through the bottom of the valley. In total, 202 canid fecal samples were randomly collected during July and August 2011, 2012 (109 in 2011 and 93 in 2012, Fig. 1). Each fecal sample was stored separately in a 50 mL centrifuge tube with 75 % ethanol. For safety reasons, all fecal samples were kept for at least 3 weeks at –80°C to inactivate *Echinococcus* eggs before further processing (Raoul *et al.*, 2001). DNA was extracted from each sample using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. Red foxes (*V. vulpes*), although rare, live sympatrically with Tibetan foxes in our research area. Thus, we followed a restriction enzyme protocol (Jiang *et al.*, 2011) to ensure that only Tibetan fox fecal samples entered the microsatellite DNA analysis. For Tibetan fox fecal samples, eleven microsatellite loci (Table 1) were amplified using the polymerase chain reaction (PCR). Reactions contained 2.5 µL of extracted DNA, 7.5 µL of Ex Taq (TaKaRa Ex Taq 1.25 U/25 µL, 0.4 mmol/L of each dNTP, 4 mmol/L magnesium salt, Takara Bio, Dalian, China), 0.6 µmol/L of the forward and reverse primers, 3.2 µL of distilled water and 0.6 µL of bovine serum albumin (Takara Bio, Dalian, China), in a total volume of 15 µL. The forward primer was modified at the 5' end with TET, FAM or HEX fluorescent labels (Sangon Biotech, Shanghai, China). Amplification conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C – 60°C (see Table 1 in detail) for 30 s, extension at 72°C for 45 s, and a final extension step of 72°C for 10 min. A Bio-rad DNA Engine PTC – 200 instrument was used (Bio-Rad, Hercules, USA).

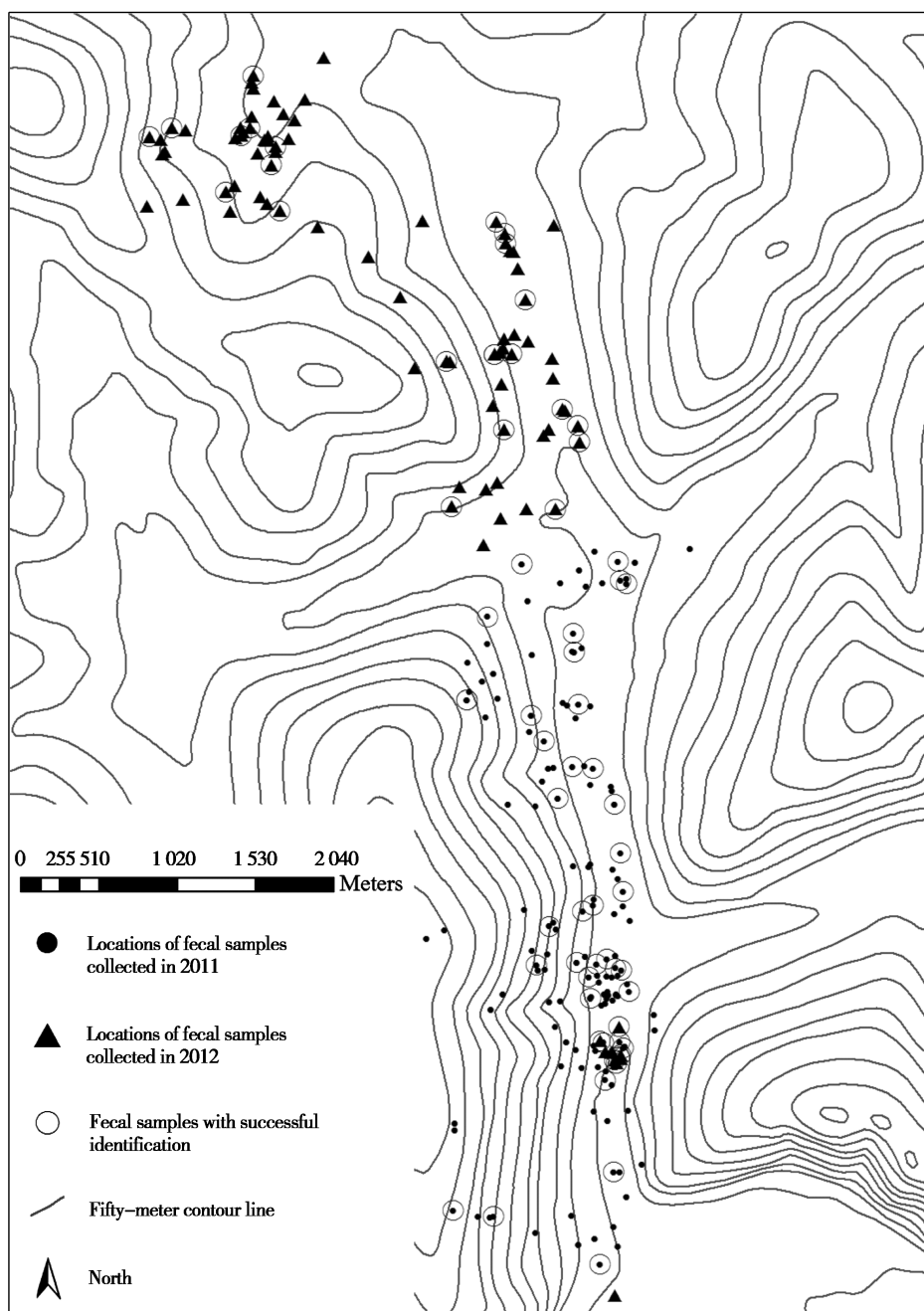


Fig. 1 Spatial distribution of fecal samples collected in 2011 and 2012

The PCR products were separated on 2% agarose gels, which were stained with GelRed nucleic acid stain (BioLum, USA) to confirm successful amplification. The products were then analyzed using an ABI 3730 XL automatic sequencer (Applied Biosystems, Beijing, China) and the Genemapper 4.0 software (Applied Biosystems, Beijing, China).

To reduce errors in genotype identification, we applied a protocol to type each locus four times (Taberlet *et al.*, 1996; Bellemain *et al.*, 2005). For each sample, if all four replicates yielded only one fragment, the microsatellite genotype of this locus was

scored as homozygous. If two fragments were detected in at least two replicates, we judged this locus as heterozygous. If the sample did not conform to any of these scenarios it was excluded from further analysis.

In addition, 21 Tibetan fox tissue samples were also tested. DNA samples had previously been prepared by Li *et al.* (2011). The copro-DNA analysis process, as described above, was used to test the quality and characteristics of the 11 microsatellite loci. The GenePop version 4.0 software (Laboratoire de Genetique Environment, Montpellier, France) was used to perform exact tests for Hardy-Weinberg equilibrium

(*HWE*) and linkage disequilibrium (*LD*).

For all samples, the number of alleles (*N*), polymorphism information content (*PIC*) values, expected heterozygosity (*He*), and observed heterozygosity (*Ho*) were calculated using the Excel-Microsatellite-Toolkit (Shaibi, 2008). The efficiency of the 11 microsatellite loci, in terms of ability to differentiate between fecal samples, was evaluated using GIMLET V. 1. 3. 3 (Valiere, 2002). The probability of identity (*PI*) values were calculated for unrelated individuals and full siblings, on the basis of the copro-DNA microsatellite data.

3 Results

Of the 109 fecal samples collected in 2011, copro-DNA was successfully extracted from 77 (70.6%) samples. Sixty-eight of them were from Tibetan foxes, whilst nine were from red foxes. Of the 93 fecal samples collected in 2012, copro-DNA was successfully

extracted from 61 (65.6%) samples. Sixty of these samples were from Tibetan foxes and one was from a red fox. Eleven microsatellite primer pairs consistently gave PCR products of the expected size from both the copro-DNA and tissue-DNA. The number of alleles (*N*), expected heterozygosity (*He*), observed heterozygosity (*Ho*) and *PIC* information for each microsatellite locus are listed and compared between the copro-DNA and tissue-DNA in Table 1. Generally, microsatellite loci in tissue-DNA gave higher values for these microsatellite parameters, compared with copro-DNA, apart from locus P03 (Table 1). Although no significant departure from pairwise linkage disequilibrium (*LD*) was observed in tissue-DNA samples, loci CPH8 ($P < 0.001$) and P02d ($P = 0.0007$) both showed significant departures from Hardy-Weinberg equilibrium (*HWE*) after a sequential Bonferroni correction (Bonferroni *P* value for significance, $P < 0.0045$).

Table 1 Characterization of 11 microsatellite loci in copro-DNA and tissue-DNA of Tibetan foxes

Locus		Repeat sequence	Size range(bp)	Ta (°C)	<i>N</i>	<i>He</i>	<i>Ho</i>	<i>PIC</i>	Amplification su ccess ate ^a	GenBank accession number/reference
P03	tissue	(GT)14	192 – 208	60	6	0. 69	0. 43	0. 6400	–	HQ452342 / Li <i>et al.</i> , 2011
	feces				7	0. 74	0. 77	0. 6921	0. 926	
CXX172	tissue	(TG)12	155	58	6	0. 75	0. 71	0. 7105	–	NC_006619 / Ostrand er, 1993
	feces				6	0. 74	0. 68	0. 6696	0. 926	
CPH6	tissue	(CA)19	107 – 136	58	5	0. 71	0. 57	0. 6651	–	Fredholm and Winte- ro, 1995
	feces				6	0. 75	0. 68	0. 7009	0. 779	
CPH8	tissue	(GT)18	186 – 200	58	10	0. 76	0. 95	0. 7257	–	Fredholm and Winte- ro, 1995
	feces				7	0. 80	0. 62	0. 7623	0. 750	
P01i	tissue	(GT)19	164 – 232	56	12	0. 81	0. 81	0. 7942	–	HQ452352 / Li <i>et al.</i> , 2011
	feces				5	0. 67	0. 38	0. 6315	0. 647	
P08	tissue	(GT)17	170 – 210	54	12	0. 81	0. 71	0. 7909	–	HQ452343 / Li <i>et al.</i> , 2011
	feces				6	0. 67	0. 30	0. 6443	0. 632	
P02 d	tissue	(GA)6...	124 – 144	54	8	0. 70	0. 43	0. 6595	–	HQ452345 / Li <i>et al.</i> , 2011
	feces	(GT)19			5	0. 69	0. 23	0. 5496	0. 412	
CPH1	tissue	(GGT)11	135 – 144	52	5	0. 71	0. 57	0. 6651	–	Fredholm and Winte- ro, 1995
	feces				5	0. 74	0. 43	0. 6771	0. 397	
DB1	tissue	(CA)22	141 – 159	58	9	0. 83	0. 81	0. 8114	–	W_003726108/ Holmes <i>et al.</i> , 1993
	feces				4	0. 69	0. 20	0. 6299	0. 353	
P05 h	tissue	(GT)20...	310 – 336	56	8	0. 76	0. 81	0. 7364	–	HQ452350 / Li <i>et al.</i> , 2011
	feces	(GA)10			4	0. 66	0. 17	0. 5938	0. 250	
AHT – 142	tissue	(TG)	133 – 148	58	7	0. 75	0. 90	0. 7169	–	DQ118707 / Wandel- er, 2006
	feces	16 ,18			5	0. 76	0. 40	0. 7325	0. 176	
Mean	tissue					0. 76	0. 73			
	feces					0. 72	0. 45			

Ta, annealing temperature of the primer pairs; *N*, number of genotypes; *He* and *Ho*, expected heterozygosity and observed heterozygosity; *PIC*, polymorphism information content; a, the amplification success rate of each locus in 128 fecal DNA samples (68 in 2011 and 60 in 2012)

All 21 tissue-DNA samples were unequivocally identified using the 11 microsatellite loci. The overall copro-DNA *PI* values were low ($PI_{biased} = 1.283 \times 10^{-11}$, $PI_{sibs} = 7.572 \times 10^{-5}$). However, the amplification success rate of each microsatellite locus in copro-DNA samples was quite different ranging from 0.926 to 0.176. We then sorted the loci according to their amplification success rates from the highest to the lowest and found *PI* values of the first six loci with amplification success rates above 60% (i.e., P03, CXX172, CPH6, CPH8, P01i, P08, Table 1) were already low enough ($PI_{biased} = 2.775 \times 10^{-7}$, $PI_{sibs} = 3.606 \times 10^{-3}$; Fig. 2) for individual identification. Thus the individual identification standards were devised as follows: (1) only copro-DNA samples with successful amplification from at least the first six microsatellite loci were used for further analysis; (2) when all alleles were identical between two samples, the samples were considered to originate from the same individual; (3) in a conservative approach, if just one allele mismatch was observed between two samples, they were also judged to originate from the same individual (Bellemain *et al.*, 2005). Thirty-eight (55.9%, 38/68) Tibetan fox copro-DNA samples, from the 2011 collection, met the standard criteria. Thirty unique genotypes were identified and thus were judged as individuals. In 2012, 28 samples (46.7%, 28/60) met the standard criteria and 21 foxes (i.e., unique genotypes) were identified.

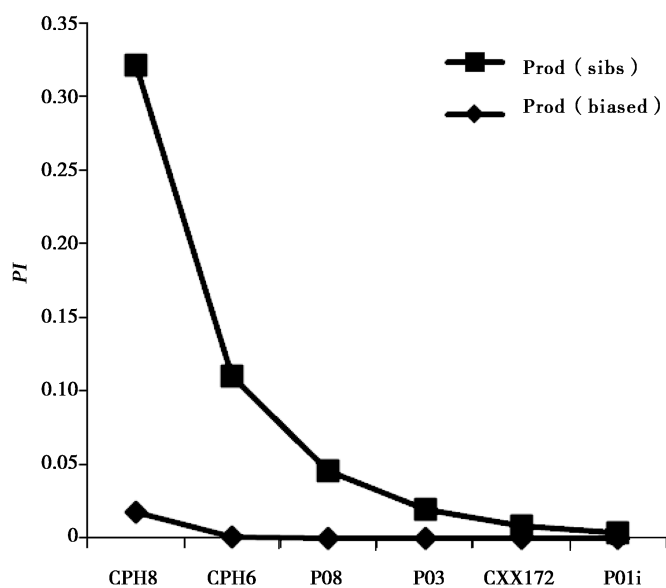


Fig. 2 Multi-loci *PI* in increasing order of single-locus values, the first locus is the most informative locus

4 Discussion

In our previous study, 15 Tibetan fox microsatellite loci were found using tissue samples (Li *et al.*, 2011), among which only five were amplified successfully and repeatedly from copro-DNA samples in this study. Jiang *et al.* (2011) reported that the low quality of copro-DNA can significantly influence DNA amplification. Therefore, effectiveness of loci developed using high quality template DNA samples must be tested before a large scaled study using copro-DNA samples. Although no significant departure from pairwise linkage disequilibrium was observed in tissue-DNA samples (Li *et al.*, 2011), loci CPH8 and P02d both showed significant departure from Hardy-Weinberg equilibrium even after a sequential Bonferroni correction. This result could have been due to the use of a small number of Tibetan fox tissue samples (21 samples), which might not reflect a true panmictic population. For example, our earlier study, using 15 loci and 36 Tibetan fox tissue samples, indicated that locus P02d did not show significant departure from Hardy-Weinberg equilibrium (Li *et al.*, 2011). Nevertheless, microsatellite loci were only used to evaluate population size in this study. CPH8 and P02d did provide genotypes for individual identification, therefore they were still used in this analysis. The overall copro-DNA *PI* values were low, which implied that the microsatellite loci are suitable as molecular markers for the individual identification of Tibetan foxes using fecal samples. Although, like reported in many other studies (Eggert *et al.*, 2003; Zhan *et al.*, 2006), some microsatellite loci had low success rates of amplification from carnivorous animal copro-DNA samples, the first six high loci (P03, CXX172, CPH6, CPH8, P01i, P08) gave sufficiently low *PI* values to be accepted for identification analysis.

Success rates of microsatellite DNA identity analysis in carnivorous species typically are low. For example, Bohling *et al.* (2011) reported an overall identification rate of 22% in canid species, and 12% in river otters (*Lontra canadensis*) (Guertin *et al.*, 2010). In contrast, the microsatellite identification rates in herbivorous species can be higher than 60% percent (Eggert *et al.*, 2003; Zhan *et al.*, 2006; Tian *et al.*, 2010). Feces of carnivorous species may degrade faster in the field than do feces of herbivorous species. As

confirmed in our previous study, the low quality of DNA and the existence of PCR inhibitors could significantly affect the success rate of copro-DNA amplification (Jiang *et al.*, 2011), which implies the importance of collecting fresh feces and sample preservation in the field.

Population information for the Tibetan fox is scarce (IUCN, 2012). Harrison *et al.* (2002) mentioned in a population study of swift foxes (*V. velox*) that the numbers of microsatellite estimated population size were overestimated because of the presence of transient individuals and poor quality DNA from feces leading to allelic drop-out and/or false alleles. However, microsatellite analysis of copro-DNA, coupled with DNA mark-recapture methods, will allow the population size to be evaluated more reasonable (Eggert *et al.*, 2003; Bellemain *et al.*, 2005). Due to logistical restrictions, we were unable to perform repeated sampling of the Tibetan foxes in this study. However, this study has provided useful microsatellite loci that are suitable for copro-DNA templates. This will make a large scale evaluation of the Tibetan fox population size feasible in the near future.

Acknowledgements: We thank colleagues from the Center of Disease Control of Shiqu County for their logistic support in the field.

References:

- Bellemain E, Swenson J E, Tallmon D, Brundberg S, Taberlet P. 2005. Estimating population size of elusive animals with DNA from hunter-collected feces: four methods for brown bears. *Conserv Bio*, **19**: 150 – 161.
- Bohling J H, Waits L P. 2011. Assessing the prevalence of hybridization between sympatric *Canis* species surrounding the red wolf (*Canis rufus*) recovery area in North Carolina. *Mol Ecol*, **20** (10): 2142 – 2156.
- Eckert J, Gemmell M A, Meslin F X, Pawlowski Z S. 2001. WHO/OIE manual on echinococcosis in humans and animals: A public health problem of global concern. Paris: World organization for animal health and world health organization.
- Eggert L S, Eggert J A, Woodruff D S. 2003. Estimating population sizes for elusive animals: the forest elephants of Kakum National Park, Ghana. *Mol Ecol*, **12**: 1389 – 1402.
- Fredholm M, Wintoro A K. 1995. Variation of short tandem repeats within and between species belonging to the Canidae family. *Mamm Genome*, **6**: 11 – 18.
- Guertin D A, Harestad A S, Ben-David M, Drouillard K G, Elliott J E. 2010. Fecal genotyping and contaminant analyses reveal variation in individual river otter exposure to localized persistent contaminants. *Environ Toxicol Chem*, **29**(2): 275 – 284.
- Harrison R L, Barr D J, Dragoo J W. 2002. A comparison of population survey techniques for swift foxes (*Vulpes velox*) in New Mexico. *Am Midl Nat*, **148**: 320 – 337.
- Holmes N G, Mellersh C S, Humphreys S J, Binns M M, Olliman A H, Curtis R, Sampson J. 1993. Isolation and characterization of microsatellites from the canine genome. *Anim Genet*, **24**: 289 – 292.
- IUCN. 2012. IUCN Red list of threatened species. Version 2012. 2. < www.iucnredlist.org >. Downloaded on 28 June 2013.
- Jiang W B, Wang X M, Li M, Wang Z H. 2011. Identification of the Tibetan fox (*Vulpes ferrilata*) and the red fox (*V. vulpes*) by copro-DNA diagnosis. *Mol Ecol Res*, **11**: 206 – 210.
- Jiang W B, Liu N, Zhan G T, Renqing P C, Xie F, Li T Y, Wang Z H, Wang X M. 2012. Specific detection of *Echinococcus* spp. from the Tibetan fox (*Vulpes ferrilata*) and the red fox (*V. vulpes*) using copro-DNA PCR analysis. *Parasitol Res*, **111**: 1531 – 1539.
- Li M, Wang X M, Jiang W B, Hua P Y, Wang Z H. 2011. Isolation and characterization of fifteen microsatellite loci in the Tibetan fox (*Vulpes ferrilata*). *J Genet*, **90**, e82 – e85.
- Li T Y, Qiu J M, Yang W, Craig P S, Chen X W, Xiao N, Giraudoux P, Mamuti W, Yu W, Schantz P M. 2005. Echinococcosis in Tibetan populations, western Sichuan Province, China. *Emerg Infect Dis*, **11**: 1866 – 1873.
- Ostrander E A, Sprague G F, Rine J. 1993. Identification and characterization of dinucleotide repeat (CA)_n markers for genetic mapping in dog. *Genomics*, **16**: 207 – 213.
- Qiu J M, Chen X W, Ren M, Luo C X, Liu D L, Liu X T, He D L. 1995. Epidemiological study on alveolar hydatid disease in Qinghai-Xizang (Tibetan) Plateau. *J Practical Parasit Dis*, **3**: 106 – 109.
- Raoul F, Deplazes P, Nonaka N, Piaroux R, Vuitton D A, Giraudoux P. 2001. Assessment of the epidemiological status of *Echinococcus multilocularis* in foxes in France using ELISA coprotests on fox faeces collected in the field. *Int J Parasit*, **31**: 1579 – 1588.
- Shaibi T, Lattorff H M G, Moritz R F A. 2008. A microsatellite DNA toolkit for studying population structure in Apismellifera. *Mol Ecol Res*, **8**: 1034 – 1036.
- Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits L P, Bouvet J. 1996. Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Res*, **24**: 3189 – 3194.
- Tian X M, Zhang M H. 2010. Population size and sex ration of wapiti (*Cervus elephus xanthopygus*) as revealed by fecal DNA. *Acta Ecol Sin*, **30**(22): 6249 – 6254.
- Valiere N. 2002. Gimlet: a computer program for analyzing genetic individual identification data. *Mol Ecol Notes*, **2**: 377.
- Wandeler P, Funk S M. 2006. Short microsatellite DNA markers for the red fox (*Vulpes vulpes*). *Mol Ecol Notes*, **6**: 98 – 100.
- Zhan X J, Li M, Zhang Z J, Goossens B, Chen Y P, Wang H J, Bruford M W, Wei F W. 2006. Molecular censusing doubles giant panda population estimate in a key nature reserve. *Curr Biol*, **16**: 451 – 452.