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ONLINE RESOURCES

Isolation and characterization of fifteen microsatellite loci in the Tibetan fox (*Vulpes ferrilata*)

MIN LI[1], XIAO MING WANG[1,2], WEI BIN JIANG[1], PAN YU HUA[1] and ZHENG HUAN WANG[1,3]*

[1]*School of Life Sciences, East China Normal University, Shanghai 200062, People's Republic of China*
[2]*Shanghai Science and Technology Museum, Shanghai 200127, People's Republic of China*
[3]*Shanghai Key Laboratory of Urbanization and Ecological Restoration, Shanghai 200062, People's Republic of China*

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Introduction

Assessing changes in local populations of wildlife species is essential to understand the dynamics of animal populations and therefore is crucial in animal ecology and wildlife conservation (Gibbs 2000). The Tibetan fox (*Vulpes ferrilata*) is an endemic plateau species distributed only in the steppes and semideserts of the Tibetan plateau in China, Nepal, and north India, at elevations between 3500 and 5300 m (Feng *et al*. 1986; Nowak 1999). To our knowledge, only a few systematic studies on the biology and ecology of this species have been published (Wang *et al*. 2007, 2008; Harris *et al*. 2008; Liu *et al*. 2009, 2010). Recently, the eastern Tibetan plateau, China, has been recognized a new endemic region for *Echinococcus* spp. with the highest prevalence of alveolar *echinococcosis* (AE) in the world (Li *et al*. 2005). AE, a lethal chronic parasitological zoonosis in north hemisphere, is caused by a canid tapeworm, *Echinococcus multilocularis* (Wang *et al*. 2008). The transmission of AE needs a stable wildlife reservoir and the Tibetan fox has been found to be the main wildlife definitive host (Qiu *et al*. 1999). To understand the epidemiology of AE in this region, the local population size of the Tibetan fox must be evaluated.

The only reports about the Tibetan fox population monitoring are from Piao (1989) and Schaller (1998). However, these early data based on observations along roadsides cannot provide much information to the present Tibetan fox population status. Therefore, IUCN stated that the population trend of Tibetan foxes is still unknown (IUCN 2010). The microsatellite genotype analysis based on the copro-DNA is a new method used increasingly in wildlife population studies (Harrison *et al*. 2002; Dallas *et al*. 2003; Zhan *et al*. 2006), which is more efficient compared with the traditional field methods. However, enough microsatellite loci must be available to perform microsatellite genotype analysis. No microsatellite locus of the Tibetan fox is available yet. Therefore, we present our study on isolation of microsatellite loci in the Tibetan fox in this paper.

Materials and methods

Tissue samples of *V. ferrilata* were obtained from 36 Tibetan foxes. Among these, seven samples were collected from Tibetan fox pelts in Ali, Tibetan Autonomic Region, 2008, and the other 29 samples were collected in Shiqu, Sichuan Province. Among the Shiqu samples, 22 were obtained from carcasses collected from 2001 to 2009, while the other seven were ear tissue samples obtained when ear-tagging during a radio-tracking study of foxes (2006–2009).

DNA was extracted from each sample using a TIANamp Genomic DNA tissue/blood kit (Tiangen, Beijing, China) according to the standard protocol in the instructions. A genomic library for CA dinucleotide repeats was constructed according to Hua *et al*. (2007). Briefly, 2–5 μg of genomic DNA extracted from *V. ferrilata* was digested with 8–24 U *Mbo*I (Takara, Dalian, China) in a volume of 50 μL at 37°C overnight, then the fragments from 250 to 750 bp were selected for DNA purification using DNA purification kit (Takara, Dalian, China) and ligated to a blunt-end adapter (SAULA: 5′-GCGGTACCCGGGAAGCTTGG-3′, SAULB: 5′-GATCCCAAGCTT CCCGGGTACCGC-3′) with T4 DNA ligase (Takara, Dalian, China). The ligation products were amplified by polymerase chain reaction (PCR) using SAULA primer. PCR recations were undertaken on a DNA thermal cycler (Bio-Rad, Hercules, USA) with an initial denaturation at 72°C for 5 min; followed by 34 cycles of 30 s at 94°C, 30 s at 67°C, 40 s extension at 72°C; and 72°C

*For correspondence. E-mail: zhwang@bio.ecnu.edu.cn.

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elongation 10 min. The amplification products were denatured and hybridized with biotin-labelled dinucleotide repeat oligonucleotides $(CA)_{15}$ probe in sodium phosphate buffer (0.5 M sodium phosphate, 0.5% SDS, pH = 7.4) overnight. The hybridized mixture was incubated with VECTREX Avidin D (Vector Laboratories, Beijing, China) at 37°C in TBS (150 mm NaCl/100 mm Tris, pH = 7.5) for 1 h, then washed thrice by TBS at different temperatures to remove unbound fragments. Finally, bound fragments were eluted with ddH_2O at 65°C for 30 min. The targeted genomic fragments were then amplified by PCR with primer SAULA, cloned into pGEM-T vector (Promega, Madison, USA), and transformed DH5α or Top10 competent cells. We identified 247 positive clones by PCR using linker primers (SAULA and SAULB) and M13 universal primers (Sangon, Shanghai, China), then sequenced with M13 primers. Primers of 63 positives (25.5% of the 247 clones) were designed with Primer PREMIER 5.0 (Premier Biosoft International, Palo Alto, USA). Thirty nine primer pairs amplified microsatellite loci and were used to screen genetic variation.

Result and discussion

To each of the 36 fox tissue samples, PCR amplification for each locus was conducted in a final volume of 15 μL containing 0.5 μM forward primer (5′ modified with FAM, HEX or TAMRA), 0.5 μM reverse primer, 50–100 ng template DNA, 0.5 mM of each dNTP, 0.5 U hotstart *Taq* DNA polymerase (Qiagen, Shanghai, China), and 1.5 μL manufacturer's PCR buffer containing 1.5 mM $MgCl_2$. Reactions were undertaken according to the following PCR procedure: 95°C initial denaturing for 5 min; 34 cycles of 95°C denaturing for 30 s, primer-specific annealing temperature (table 1) for 30 s and extension for 40 s at 72°C; final extension at 72°C for 10 min. PCR products were separated through 2% denaturing polyacrilamide gels. The fragments were detected using an ABI 3730 XL automatic sequencer (Applied Biosystems, Beijing, China) and analysed with Genemapper 4.0 (Applied Biosystems, Beijing, China).

GenePop version 4.0 (Laboratiore de Genetique et Environment, Montpellier, France) was used to calculate the

Table 1. Characterization of 15 microsatellite loci in 36 Tibetan foxes.

Locus	Primer sequence 5′-3′	GenBank accession number	Repeat sequencec	T_a (°C)	N	A	Size range (bp)	H_e	H_o
p02	GTGACGCCCAAACATCC GGCTCCTGCTCCTCTGC	GU944946	$(TG)_4\ldots(TG)_3\ldots(TG)_3$	58	3	3	140–168	0.177	0.125
p03	GAAAGCCAGGGTCCAGTA CAGAAACGGGCATCCATA	HQ452342	$(GT)_{14}$	58	11	7	192–208	0.653	0.419
p08	ATTCTGGACCAATGAGGC GGAGGGGAGGAAGGATA	HQ452343	$(GT)_{17}$	62	17	15	170–210	0.843	0.742
p09	GGTGGAGACTGCTTGGGA CCTGTGGAATAGAGCGGG	GU944947	$(CA)_7\ldots(CA)_8$	58	4	3	300–336	0.423	0.207
p02c	AGAAGCAGGATTCACACC TTCCCTCAACACTCACC	HQ452344	$(CA)_{23}$	54	14	9	180–222	0.812	0.939
p04c	GATCAAGTCCCAGGTCG CACTGAGTTAGCCAGATG	GU944948	$(GT)_{21}$	56	3	3	170–280	0.092	0.094
p02d	CTTATTGGGCTTCTTGG TTGCCTCAGCATCTCATC	HQ452345	$(GA)_6\ldots(GT)_{19}$	60	12	8	124–144	0.692	0.528
p01f	GCCTGGGAAGGAATCAAG CCTGGCTCCATCCAAACA	HQ452346	$(CA)_{17}$	64	16	10	170–230	0.857	0.778
p02h	CTGGGAAGGAAGCAAGA CATACACCCTAAGCAACTG	HQ452347	$(CA)_{17}$	58	14	7	195–207	0.793	0.800
p03h	TCTACCCATACAGCATCC GAGCCAGTGTCTATCCCT	HQ452348	$(CA)_{22}$	58	8	6	226–236	0.740	0.667
p04h	CTGCTGGAAAGAGGAAT CAGAGCCAAAGGTAGGT	HQ452349	$(CA)_{21}$	58	19	11	203–235	0.840	0.743
p05h	TCACTCCTAAGAAATCGG GCACTGGCATGAACTGG	HQ452350	$(GT)_{20}\ldots(GA)_{10}$	58	17	10	310–336	0.778	0.829
p06h	GGATGGGCAGATGGAGCA GCCTGAGCAGTTGACCTT	HQ452351	$(GT)_{20}$	58	6	6	281–313	0.487	0.627
p01i	GGGGACCTCAAGAATGT TGTCTCATCAATGCCAAG	HQ452352	$(GT)_{19}$	58	22	15	164–232	0.836	0.686
p03i	ATCTCCTCCAAGACCTCC TCCCACCCCTGATACCT	HQ452353	$(CA)_{10}\ldots(CA)_7$	58	20	9	447–463	0.867	0.914

T_a, annealing temperature of the primer pairs; N, number of genotypes; A, alleles found in fox individuals; H_e and H_o, the size range, expected heterozygosity and observed heterozygosity are given for each locus. The size range refers to the observed distribution of alleles at each locus for 36 individuals.

Table 2. Genotypes of the 36 individuals in this study for the 15 qualified microsatellite loci.

	Microsatellite locus														
Individual number	p02	p03	p08	p09	p02c	p04c	p02d	p01f	p02h	p03h	p04h	p05h	p06h	p01i	p03i
tf1	3	7	5	2	13	3	1	0	5	6	16	14	2	15	10
tf2	3	7	6	2	8	0	5	0	14	0	13	14	2	13	4
tf3	1	3	8	2	5	3	2	2	14	6	10	15	2	14	11
tf4	3	7	8	2	7	3	1	4	11	6	14	14	2	22	4
tf5	1	4	10	2	5	3	4	4	6	3	14	9	2	18	3
tf6	3	1	14	2	9	0	4	1	12	6	14	7	2	8	4
tf7	3	7	5	2	8	3	4	4	13	0	13	17	2	17	7
tf8	3	3	11	3	10	3	1	14	12	8	10	14	4	13	9
tf9	1	7	10	2	8	3	4	15	6	0	14	16	4	11	19
tf10	3	7	9	4	8	3	4	6	6	4	9	11	4	16	12
tf11	3	4	10	4	7	3	4	9	1	0	16	16	4	16	9
tf12	3	6	7	3	7	3	7	0	4	0	18	13	2	6	20
tf13	3	3	7	2	11	1	10	8	7	0	19	16	1	5	17
tf14	3	4	7	2	9	3	2	13	4	0	16	10	3	10	4
tf15a	3	8	8	2	8	3	1	16	6	6	17	6	3	18	12
tf15b	3	10	13	2	7	3	1	9	6	5	11	6	3	7	14
tf16	3	3	8	2	7	3	8	13	9	0	16	13	3	16	2
tf17	3	2	0	0	9	3	1	9	2	0	10	15	3	4	13
tf18	3	7	8	2	9	3	9	9	4	0	17	5	3	16	4
tf19	3	7	6	2	8	3	6	12	4	0	12	16	3	20	4
tf20	3	8	5	3	8	3	1	11	0	8	15	13	3	19	11
tf21	3	4	13	4	0	3	1	11	11	0	11	15	3	11	14
tf24	3	4	12	0	0	3	4	4	3	5	8	16	3	21	6
tf25	3	9	17	2	14	3	12	0	0	0	2	1	5	10	12
tf26	2	0	1	2	5	3	10	0	0	0	4	2	6	13	14
tf27	3	5	15	1	2	2	10	0	0	7	4	13	2	1	1
tf28	3	11	16	2	1	3	10	8	13	0	5	3	2	16	16
tf29	0	0	0	0	12	0	11	0	8	1	3	17	2	2	15
tf30	3	7	16	1	4	3	10	0	0	2	6	4	3	12	1
tf31	0	0	0	0	6	1	11	0	13	0	1	14	2	11	18
tf32	3	3	2	0	0	3	4	5	0	0	0	0	0	0	0
tf33	0	0	0	0	9	3	3	3	10	5	11	8	3	11	3
tf34	1	3	4	3	9	3	10	10	4	0	11	9	3	9	15
tf35	3	4	15	4	3	3	9	10	4	0	17	9	3	3	4
tf36	3	7	3	0	11	0	2	7	10	0	7	12	2	17	5
tf37	0	0	0	4	9	3	10	10	12	0	16	13	3	16	8

For each locus, each figure indicates one genotype; the figure '0' indicates that the locus in this individual was unable to amplify.

genetic diversity indices. *P* values of all the indices were corrected by Bonferroni procedure (Rice 1989). Among all the 39 loci, 15 were in Hardy–Weinberg equilibrium (Bonferroni $P > 0.0033$), and no significant linkage disequilibrium was found between all the pairs of these 15 loci (Bonferroni $P > 0.0002$). Consequently, these 15 loci qualified as identifying molecular markers. The number of alleles at these 15 loci ranged from 3 to 15. The observed heterozygosities ranged from 0.094 to 0.939, while the expected range is from 0.092 to 0.867 (table 1).

Table 2 shows all genotypes of the 36 fox individuals in this study, which demonstrated the successful identifications of all the tested Tibetan fox individuals using the 15 qualified microsatellite loci. Loci with higher number of alleles have higher diversity in genotypes (table 1). These loci are able to provide more genetic diversity information of a population, therefore are important in the population genetic study. Several fox individuals (e.g., tf29, tf31, and tf33) which were collected in a same valley in Ali showed identical results in the failure of amplifying loci p02, p03, p08, and p09 (table 2). The genetic and spatial relationship among all the individuals will be further analysed.

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