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ORIGINAL PAPER

Risks involved in fecal DNA-based genotyping of microsatellite loci in the Amur tiger *Panthera tigris altaica*: a pilot study

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Abstract In modern wildlife ecological research, feces is the most common non-invasive source of DNA obtained in the field and polymerase chain reaction (PCR) technology based on microsatellite markers is used to mine genetic information contained within. This is especially the case for endangered species. However, there are risks associated with this genotyping method because of the poor quality of fecal DNA. In this study, we assessed genotyping risk across 12 microsatellite loci commonly used in previous tiger studies using blood and fecal DNA from captive Amur tigers (*Panthera tigris altaica*). To begin, we developed an index termed the accumulated matching rate of genotypes (R_m) between positive DNA (blood samples) and fecal DNA to explore the correct genotyping probability of a certain microsatellite locus. We found that different microsatellite loci had different genotyping risks and required different PCR amplification protocols. The genotyping errors we detected altered population genetic parameters and potentially impact subsequent analyses. Based on these findings, we recommend that: (1) four loci (E7, Fca094, Pti007 and Pti010) of 12 loci are not suitable for Amur tiger genetic research because of a low R_m and difficulty reaching a stable status; (2) the R_m of the 12 microsatellite loci plateaued differently, and considering limited budgets, amplification times of some loci could be increased when using fecal samples; and (3) future genetic analysis of wild Amur tigers should be corrected by genotyping error rates ($1 - R_m$).

Keywords Amur tiger · Genotyping risk · Microsatellite · Non-invasive sample

Introduction

The emergence of molecular genetic marker-based technologies presents opportunities for mining information from feces and hair, materials that are both commonly collected in the field but made little use of in traditional monitoring methods. Non-invasive sampling of feces and hair and relevant genetic analyses have become popular in wildlife monitoring and management (Taberlet and Luikart 1999), especially for endangered species (Woodruff 1993). The quality and quantity of DNA extracted from fecal samples are more or less influenced by diet composition (Stenglein et al. 2010). Research shows that an interaction exists between the complexity of diet composition and the probability of false allele amplification in polymerase chain reaction (PCR), and compared with herbivores the quality and quantity of DNA extracted from carnivores is poorer because of complicated scat composition (Panasci et al. 2011).

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As a rare and elusive carnivore species, it is difficult to collect samples like blood or tissue from Amur tigers in the field, but feces are easily obtained during wildlife surveys. Routine procedures for fecal analysis include isolation of genomic DNA and amplification of a panel of microsatellite loci and bio-informative analyses. However, due to poor quality and small quantities of template DNA resulting from degradation or contamination (Bradley et al. 2000; Regnaut et al. 2006), microsatellite loci amplification and genotyping can greatly impact subsequent analyses and have wildlife management implications. Although some population genetic studies of Amur tiger have used feces as a DNA source (Rozhnov et al. 2009), their use has not been tested through scientific pilot studies that help to reduce genotyping errors associated with template DNA of poor quality (Arandjelovic et al. 2009).

Factors affecting microsatellite analysis of Amur tigers include efficiency of DNA isolation and PCR. The efficiency of DNA isolation largely depends on storage methods (Nsubuga et al. 2004), season of collection (Hájková et al. 2006), and age of the scat (Murphy et al. 2007; Santini et al. 2007). The efficiency of PCR mainly depends on the nature of primer sequences, amplification fragment length, secondary structure, melting temperature and nucleotide composition (Sambrook et al. 2001). Software based estimation of genotyping errors is based on statistical models sensitive to sample size; however, the population size of wild Amur tigers is small, and the chance for sampling different tiger individuals is biased and software may not be effective. In this research, we improved the efficiency of DNA isolation and only studied the effect of microsatellite-based PCR protocols on the genetic analysis of Amur tigers. We compared the accordance rate of genotyping efficiency, individualization and inter-individual genetic relationships between blood and fecal samples for 10 captive Amur tigers (*Panthera tigris altaica*) with 12 microsatellite loci utilized in wild tiger population monitoring (Bhagavatula and Singh 2006; Rozhnov et al. 2009). Specifically, we quantified the variation in genotyping risk among the 12 microsatellite loci used for individualization and genetic relationship estimation, and further, whether among-loci variation was influenced by repeated PCRs when fecal DNA was used. Our pilot study and the results will provide a frame of reference for conservation programs designed on DNA analyses of wild Amur tigers.

Materials and methods

Samples

Feces and peripheral blood samples of 10 adult Amur tigers were collected in Heilongjiang Amur Tiger Park in November 2013. Blood samples were collected by veterinarians three months after birth for establishing genetic lineage. These blood samples were anticoagulated with sodium citrate (3.8%) and temporarily stored at 4 °C until DNA was extracted. Feces were collected from the same group of tigers using plastic bags within 12 h after defecation and temporarily stored in an ice bag and then transferred to a refrigerator (−20 °C) before DNA extraction.

DNA extraction

DNA of blood samples was extracted using the standard phenol and chloroform method (Sambrook et al. 1989). DNA of fecal samples was extracted using the modified QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) as described by Zhang et al. (2009) and with optimized preparation work as follows. About 5 g of feces was peeled off from the surface of a fecal pellet and deactivated in 100% ethanol with volume a ratio of 1:1 at room temperature for 12 h. Feces and ethanol were then vortexed at 2200 rpm for 3 min. The mixture was then filtered through a piece of sterile gauze. The filtrate was collected and centrifuged at 3500 rpm for 15 min and pellets at the bottom were transferred to a new tube for DNA extraction. The quality and quantity of DNA extracted from blood and feces were evaluated using routine agarose (1.0%) electrophoresis with quantification molecular markers.

Microsatellite analysis

A panel of 12 microsatellite loci was selected from former studies on tiger monitoring and individualization, including D10, Fca43, Fca304, E21B, E6 and E7 for wild tigers (Bhagavatula and Singh 2006; Rozhnov et al. 2009), and FCA391, FCA441, Fca094, Fca152, Pti007 and Pti010 for captive tigers (Xu et al. 2005; Menotti-Raymond et al. 1999; Zhang et al. 2003). Primer sequences, repeating motifs, annealing temperatures and expected allele size ranges of these microsatellite loci are shown in Table 1. The 5′ end of each forward primer was labeled with fluorescent dye (e.g., 5-FAM, TAMRA and HEX).

PCR was carried out in a 20 μL system containing 1× PCR buffer containing 50 mM Tris–HCl (pH 8.0), 25 mM KCl, 0.1 mM EDTA, 1 mM dTT; 0.4 mM each of 4 dNTP (TOYOBO), 0.2 μM each of forward and reverse primer, 0.4 U units of KOD FX Neo DNA polymerase (TOYOBO) and about 80 ng of genomic DNA. PCR amplification was performed on a Model 9700 Thermocycler (Perkin-Elmer) using the following condition: 1 cycle of 2 min at 94 °C, 35 cycles of 98 °C for 20 s, annealing temperature (i.e. Tm) (52–62 °C, Table 1) for 30 s, 68 °C for 20 s, and 1

Table 1 Characterizations and conditions of polymerase chain reaction for 12 microsatellite loci of Amur tiger

Microsatellite locus	Primers (5′–3′)	Repeat motif	Annealing temperature (°C)	PCR product size range (bp)
E6	CCTGGGGATAATAAAACTAGTA CATGAATGAATCTTTACACTGA	$(TAA)_{11}$	58	147–162
E21B	GCGATAAAGGCTGGCAGAGG CTTTGAGGGTCTGTTCTACTGTGA	$(CA)_{21}$	62	154–168
D10	CCCTCTCTGTCCCTCCCTTG GCCGTTTCCCTCATGCTACA	$(GT)_{14}$	62	134–150
E7	GCCCCAAAGCCCTAAAATAA GCATGTCGGACAGTAAAGCA	$(CA)_{11}CG(CA)_4$	58	136–156
Fca304	TCATTGGCTACCACAAAGTAGG CTGCATGCCATTGGGTAAC	$(GT)_{17}(GG)_1(GT)_6$	58	120–134
Fca043	GAGCCACCCTAGCACATATACC AGACGGGATTGCATGAAAAG		58	116–130
FCA391	GCCTTCTAACTTCCTTGCAGA TTTAGGTAGCCCATTTTCATCA	$(ATGG)_{10}(GATA)_{11}(TAGA)_2TGA(TAGA)_1$	55	190–230
Fca152	TTTAGTCAGCTTAGGCTTCCA CTTCCCAGCTTCCAGAATTG	$(AC)_{21}$	58	129–147
Pt i007	ATCAGGAGTTCTATCACC CATGATTAGGGAGTTGAG	$(AC)_{16}$	52	139–193
FCA441	ATCGGTAGGTAGGTAGATATAG GCTTGCTTCAAAATTTTCAC	$(ATAG)_9(GTAG)_1(ATAG)_2AG(ATAG)_1$	58	130–168
Fca094	TCAAGCCCCATTTTACCTTC CACCTGAGCCAAAGGCTATC	$(GT)_{19}(AG)_{22}$	58	193–215
Pt i010	GGGACAACTGAGAGAAGA CAAGATATGTTCTCAGACTG	$(AC)_8$	58	118–134

cycle of 68 °C for 20 min. A positive and two negative controls were included for each set of amplification.

PCR products were analyzed with an ABI 3100 Automated DNA Sequencer (Applied Biosystems) and genotyping data collected using GeneScan3.1 and Geno-Typer 3.1 (Applied Biosystems). Amplification and genotyping were repeated 7 times for fecal DNA, while for blood DNA, homozygotes whose signals were not perfect were reamplified twice to confirm the genotypes as described by Liu et al. (2013).

Evaluation of genotyping correctness at each locus

The blood genotype of each microsatellite locus for each tiger was regarded as 100% correct and used as the standard for our evaluation of genotyping correctness of fecal DNA amplifications. A genotype from a fecal sample at a locus was determined as 'correct genotyping' when it matched the genotype of blood DNA of the same individual, and as 'false genotyping' when it failed to match the blood DNA genotyping result. For each locus we calculated the accumulative matching rate of genotypes (R_m) between blood and fecal samples for each locus as the total number of correctly genotyped tigers divided by the total number of genotyping trials:

$$R_m = \frac{\sum_{i=1}^{m} N_i}{nm}$$

where, R_m is the cumulative matching rate of genotypes between blood and feces when PCR is repeated m times, n the number of tigers ($n = 10$), and N_i is the number of tigers with correct genotyping at the ith PCR. The genotyping risk of fecal samples for each locus is then expressed as $1 - R_m$.

Evaluation of population genetic parameters using fecal samples

Population genetic parameters were computed for blood samples and fecal samples using POPGENE v1.32 (Yeh et al. 2001), including observed heterozygosity (H_o), expected heterozygosity (H_e), the number of alleles (A), allelic frequency, effective number of alleles (A_a), and polymorphism information content (PIC). Discrimination

power (DP) and the exclusion probability of paternity (EPP) were computed using CERVUS v3.0 (Marshall et al. 1998). Pearson's bivariate correlation between genotyping risk ($1 - R_m$) and absolute differences of these parameters between blood and feces groups were calculated using SPSS 13.0. Pairwise relatedness of individuals (r_R) was computed by Coancestry v1.0.12 based on genotyping data from blood and feces. Linear regression of r_R between blood and fecal samples was analyzed using SPSS 13.0 (SPSS, Inc., Chicago, USA).

Results

Evaluation of genotyping correctness at each locus

For each fecal sample and microsatellite marker, PCR was performed 7 times and the matching rate for genotypes varied from 30–100%, averaging 71% across the 12 loci. According to the association between the cumulative matching rate of genotypes (R_m) and number of PCR repeats, for 8 loci R_m plateaued by the third PCR and for 11 loci, R_m plateaued by the fifth PCR (Fig. 1). Pti010 had a fluctuating R_m, indicating an unstable allele amplification efficiency for each PCR round.

R_m at the plateau (e.g. after the fifth PCR) varied significantly among loci (Fig. 1; Table 2). E6 had the greatest plateaued cumulative R_m at 0.871, while Pti007 had the lowest value at 0.357. The mean plateau cumulative R_m was 0.710 ± 0.139 for the 12 microsatellite loci.

Effects of genotyping error on estimation of population genetic parameters

For each fecal sample and microsatellite locus, PCR was performed 7 times. The fecal genotype of each locus was regarded as *correct* when it matched the genotype obtained from a blood sample of the same tiger 4 times or more. Characteristics of the 12 microsatellite loci and comparisons between fecal and blood samples are listed in Table 2.

The number of alleles (A) observed per locus ranged from 3 to 6 for blood samples ($\bar{x} = 4.25$), and from 3 to 5 for fecal samples ($\bar{x} = 4.08$). Allelic frequency was different between blood and feces for all loci except E6. This influenced population genetic parameters based on allelic frequencies. Parameter values calculated from blood samples and fecal samples were differed insignificantly (t test, all $p > 0.05$) for mean effective number of alleles (mean $A_a = 2.629$ for blood; mean $A_a = 2.439$ for feces), mean expected heterozygosity (mean $H_e = 0.616$ for blood; mean $H_e = 0.597$ for feces), PIC (mean PIC = 0.543 for blood; mean PIC = 0.518 for feces), DP (mean DP = 0.228 for blood; mean DP = 0.245 for feces), and EPP (mean EPP = 0.470 for blood; mean EPP = 0.502 for feces). Pearson's bivariate correlation test showed that genotyping risk ($1 - R_m$) was not correlated with absolute differences in population parameters between blood and fecal samples (H_e: a = 0.431, $p = 0.161$; A_a: a = 0.354, $p = 0.259$; PIC: a = 0.411, $p = 0.184$; DP: a = 0.385, $p = 0.217$; and EPP: a = 0.365, $p = 0.244$).

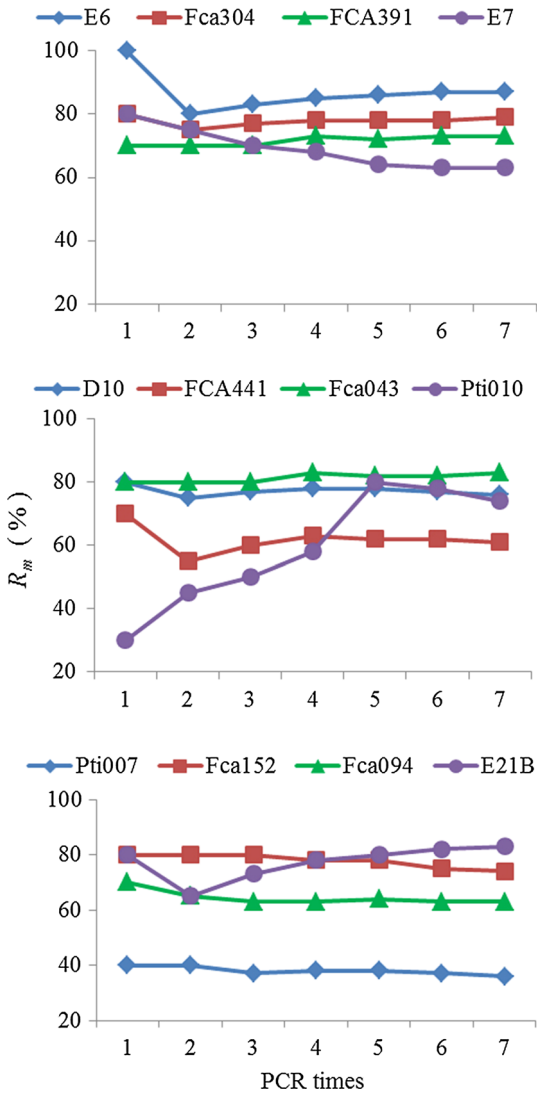

Fig. 1 Association between accumulative matching rate of genotypes (R_m) and repeated PCR for each locus

We computed pairwise relatedness (r_R) among individuals using blood and fecal genotypes. The range of r_R among individuals using blood genotypes was −0.36 to 0.22, and the range of r_R among individuals using fecal genotypes was −0.41 to 0.23. The regression coefficient should be 1.0 if r_R values of a given individual pair generated from blood and feces are equal, demonstrating that genotyping errors for feces do not influence the estimation of pairwise relatedness. Our results showed that R^2 was

Table 2 Characteristics of 12 microsatellite loci and comparison between fecal and blood samples in a captive tiger population (n = 10)

Locus	Allelic frequency							H_o	H_e	A	N_e	PIC	DP	EPP	R_m (%)	$1 - R_m$ (%)
E6	Alleles	150	153	159											87.1	12.9
	Blood	0.650	0.250	0.100				0.700	0.532	3	2.020	0.443	0.308	0.602		
	Feces	0.650	0.250	0.100				0.700	0.532	3	2.020	0.443	0.308	0.602		
E21B	Alleles	156	158	166											82.9	17.1
	Blood	0.750	0.150	0.100				0.500	0.426	3	1.681	0.368	0.391	0.655		
	Feces	0.650	0.250	0.100				0.500	0.532	3	2.020	0.443	0.308	0.602		
D10	Alleles	136	138	144	146	148									75.7	24.3
	Blood	0.150	0.050	0.050	0.100	0.650		0.600	0.568	5	2.174	0.508	0.244	0.484		
	Feces	0.200	0.000	0.050	0.050	0.700		0.500	0.490	4	1.869	0.421	0.331	0.600		
E7	Alleles	138	142	148	152										62.9	37.1
	Blood	0.100	0.250	0.550	0.100			0.900	0.647	4	2.597	0.562	0.201	0.458		
	Feces	0.100	0.100	0.700	0.100			0.600	0.505	4	1.923	0.450	0.300	0.548		
Fca304	Alleles	124	126	130	132	136									78.6	21.4
	Blood	0.200	0.200	0.400	0.150	0.050		0.700	0.774	5	3.774	0.694	0.111	0.310		
	Feces	0.150	0.300	0.400	0.100	0.050		0.600	0.753	5	3.509	0.668	0.128	0.342		
Fca043	Alleles	119	121	125	127	129									82.9	17.1
	Blood	0.050	0.100	0.200	0.050	0.600		0.700	0.616	5	2.410	0.544	0.213	0.456		
	Feces	0.050	0.100	0.200	0.100	0.550		0.800	0.668	5	2.740	0.595	0.173	0.401		
FCA391	Alleles	198	202	206	210	218	222								72.9	27.1
	Blood	0.050	0.450	0.100	0.250	0.050	0.100	0.900	0.747	6	3.448	0.671	0.123	0.319		
	Feces	0.000	0.400	0.050	0.350	0.100	0.100	0.800	0.732	5	3.279	0.643	0.145	0.371		
Fca152	Alleles	131	137	139	141	143									74.3	25.7
	Blood	0.100	0.000	0.150	0.100	0.650		0.400	0.563	4	2.151	0.498	0.253	0.505		
	Feces	0.050	0.050	0.200	0.000	0.700		0.500	0.490	4	1.869	0.421	0.331	0.600		
Pti007	Alleles	141	175	177	191	193									35.7	64.3
	Blood	0.150	0.700	0.150	0.000	0.000		0.600	0.490	3	1.869	0.420	0.331	0.610		
	Feces	0.000	0.600	0.100	0.050	0.250		0.800	0.595	4	2.299	0.509	0.245	0.517		
FCA441	Alleles	136	148	152	156	160									61.4	38.6
	Blood	0.000	0.400	0.250	0.250	0.100		0.700	0.742	4	3.390	0.652	0.141	0.379		
	Feces	0.050	0.450	0.200	0.250	0.050		0.700	0.726	5	3.226	0.640	0.146	0.376		
Fca094	Alleles	197	201	203	205	207	211								62.9	37.1
	Blood	0.150	0.150	0.100	0.150	0.400	0.050	0.700	0.800	6	4.167	0.730	0.088	0.250		
	Feces	0.200	0.100	0.000	0.150	0.550	0.000	0.600	0.658	4	2.667	0.578	0.188	0.436		
Pti010	Alleles	124	126	128											74.3	25.7
	Blood	0.150	0.150	0.700				0.400	0.490	3	1.869	0.420	0.331	0.610		
	Feces	0.200	0.100	0.700				0.400	0.484	3	1.852	0.410	0.341	0.624		

only 0.491, although the linear trend was significant ($p < 0.001$; Fig. 2). This violated the prediction and suggests that genotyping errors in fecal samples influenced the estimation of individual genetic relatedness.

Discussion

The efficiency of genetic analysis using non-invasive samples, particularly feces, is often restricted by microsatellite genotyping errors, leading to inaccurate estimation of population genetic parameters and unreasonable conservation and management strategies (Garshelis et al. 2008). Some claim that improvement of genotyping accuracy largely depends on effective sample preservation (Roon et al. 2003); however, the quality of feces collected from the wild cannot be guaranteed because of exposure to degradation factors prior to collection (Brinkman et al. 2010; Nsubuga et al. 2004). Effective preservation and DNA extraction would have limited positive impact on the analysis of poor quality feces (Huber et al. 2003). Although multi-tube PCR could reduce the effects of genotyping

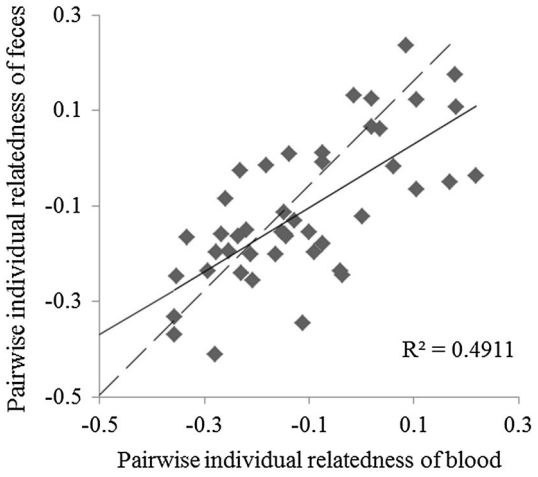

Fig. 2 Comparison of *Lynch and Ritland-estimator* (r_R) of pairwise relatedness among individuals between blood and feces genotypes. *Dotted line* represents the equation y = x

errors (Taberlet et al. 1996), we chose ordinary PCR so that we could effectively control factors (such as reaction temperature) for each locus. Therefore, fecal samples have to be discarded when they are inferior in quality, and laboratory procedures need to be optimized to improve overall genotyping accuracy for samples chosen for analysis. The first step in optimizing laboratory procedures is to assess genotyping risk and any possible impacts on subsequent analyses.

We identified two sources of genotyping error resulting from laboratory procedures, viz. PCR amplification and the nature of the microsatellite fragment. Our index cumulative matching rate of genotypes (R_m) contained information about both sources of error.

For PCR amplification, the R_m of 12 microsatellite loci plateaued after 3–5 PCRs. This suggests that the amplification efficiency of alleles fluctuated due to a poor effective template, and each PCR had a certain genotyping risk. The quality of fecal samples used in this study was good overall because they were collected within 12 h after defecation and stored at −20 °C. In northeast China, cold weather and deep snow can also preserve samples quite well in winter in the field. However, PCR repeats may be required to achieve R_m plateau in fecal samples of poor quality collected in other seasons.

R_m also demonstrated that different microsatellite loci have different genotyping accuracies, even when the same batch of samples is used (Table 2; Fig. 1). This suggests that each locus might have its own tolerance limit for poor DNA templates. Perhaps uneven degradation patterns of DNA molecules means that some parts are more sensitive to degrading factors (e.g. bacterial nuclease) (Deagle et al. 2006) and therefore for the same DNA sample, the effective DNA template content would be lower for some microsatellite loci. Or perhaps secondary structures at a certain primer site differentially impact annealing efficiency (Nazarenko et al. 2002). Even though annealing can be improved by optimizing annealing temperature or PCR components, such effects cannot be completely removed. Either way, unlike previous reports that assessed overall PCR repeats for a whole panel of microsatellite loci (He et al. 2011), our results suggest that genotyping risk should be assessed for each locus.

Genotyping errors did alter population parameters such as A_a, H_e, PIC, DP and EPP (Table 2), but not in a statistically significant way, and there was no significant association with genotyping risk ($1 - R_m$). However, drawing the conclusion that genotyping errors do not impact the estimation of population genetic parameters may be premature. For example, when looking at R_m values for the 12 microsatellite loci, the lowest R_m was 0.357. We performed PCR 7 times for 10 tigers and according to the R_m equation there should be 24.99 tigers correctly genotyped, almost 3 times the real number of tigers we used (n = 10). This roughly demonstrates that a tiger could be correctly genotyped 3 times when PCR is performed 7 times. For other loci with higher R_m values, genotyping would be more reliable; however, the quality of feces varied among tigers and coverage of 3 times might not have covered all tigers. This means that not all tigers would be correctly genotyped and variation in feces quality requires more microsatellite loci with high R_m values, and vice versa.

In conclusion, genotyping risks for microsatellite loci from fecal DNA are derived from the PCR process and the nature of the microsatellite. Our pilot study designed for Amur tigers was part of a current Amur tiger population monitoring program, and the result will be applied to wild Amur tiger fecal DNA analyses in order to conduct secure genotyping. We recommend that (1) four loci (E7, Fca094, Pti007 and Pti010) are not suitable for genetic research in Amur tigers because of low R_m values; (2) the R_m of 12 microsatellite loci plateaued differently, and considering limited budgets the amplification times of a few loci should be increased when loci are used for wild fecal samples; and (3) genetic analysis of wild Amur tigers should be corrected using the genotyping error rate ($1 - R_m$).

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