UNITED NATIONS
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EAST AND NORTH-EAST ASIA OFFICE

Review meeting on the NEASPEC Project
"Study on Transborder Movement of Amur Tigers and Leopards using Camera Trapping and Molecular Genetic Analysis"

Molecular Genetic Analysis Method of Amur Tiger and Amur Leopard

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September 15, 2015

Harbin, China



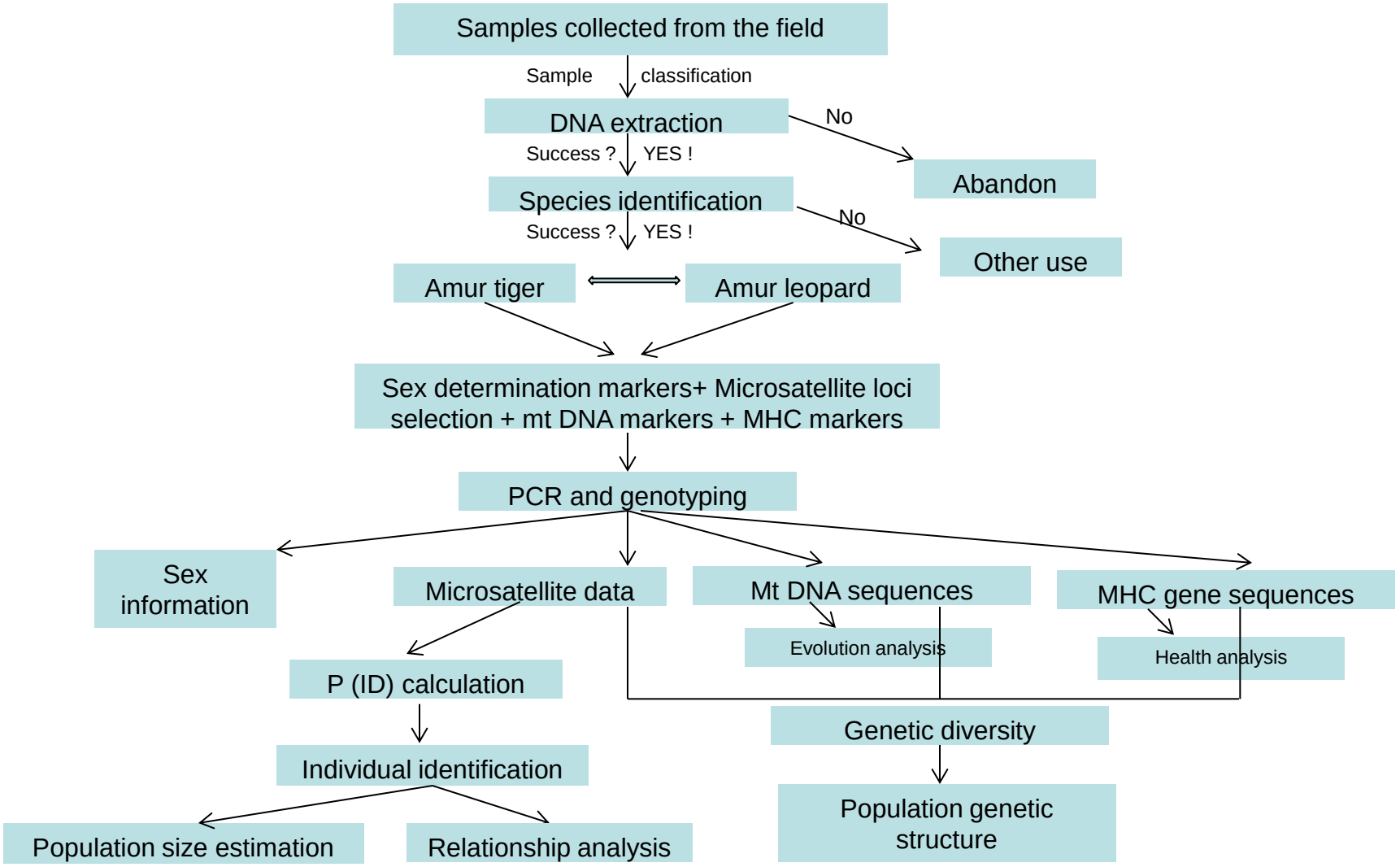
Outline

- Part 1 Key questions
- Part 2 What have ‘THEY’ done ?
- Part 3 Will ‘good’ be ‘perfect’ ?
- Part 4 Recommendations
- Part 5 Our methods

Part 1 Key questions related with Amur tiger and Amur leopard conservation and management practice

- ✓ What is that ? (Species identification)
- ✓ How many are there ? (individual identification, population size)
- ✓ How are they ? (Sex ratio, Genetic diversity and structure , Health condition)
- ✓ What is good ? (methods commonly used)

Data analysis framework



Part 2 What have ‘THEY’ done to resolve these questions ?

2.1 What is that ?

2.2 How many are there ?

2.3 How are they ?

2.4 what is good ?

OR?

OR?

2.1 What is that ?

Species identification

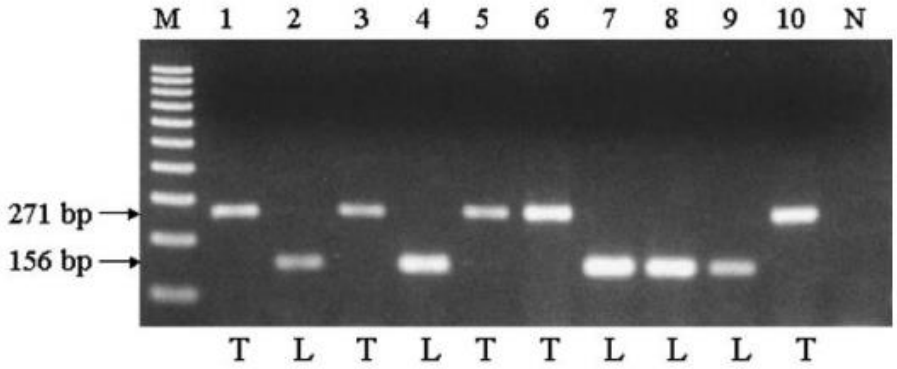


Figure 1. Examples for species identification from faecal samples. *Panthera tigris altaica* specific fragments (271 bp) are observed in lanes 1, 3, 5, 6 and 10. *Panthera pardus orientalis* specific fragments (156 bp) are observed in lanes 2, 4, 7, 8 and 9. Lane M: 100 bp ladder maker; Lane N: PCR negative control. T and L denote tiger and leopard, respectively.

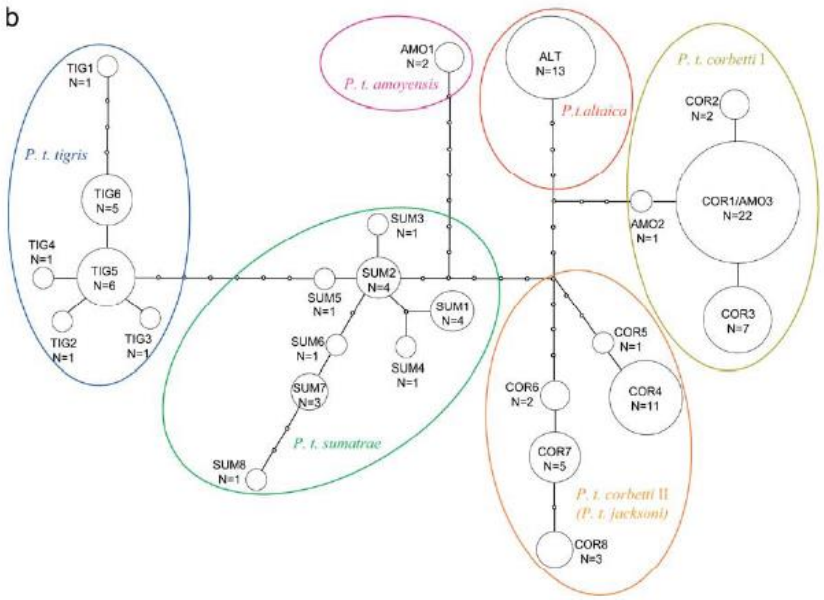
Sugimoto, T. *et al*. (2006). Species and sex identification from faecal samples of sympatric carnivores, Amur leopard and Siberian tiger, in the Russian Far East. *Conservation Genetics*, *7*(5), 799-802.

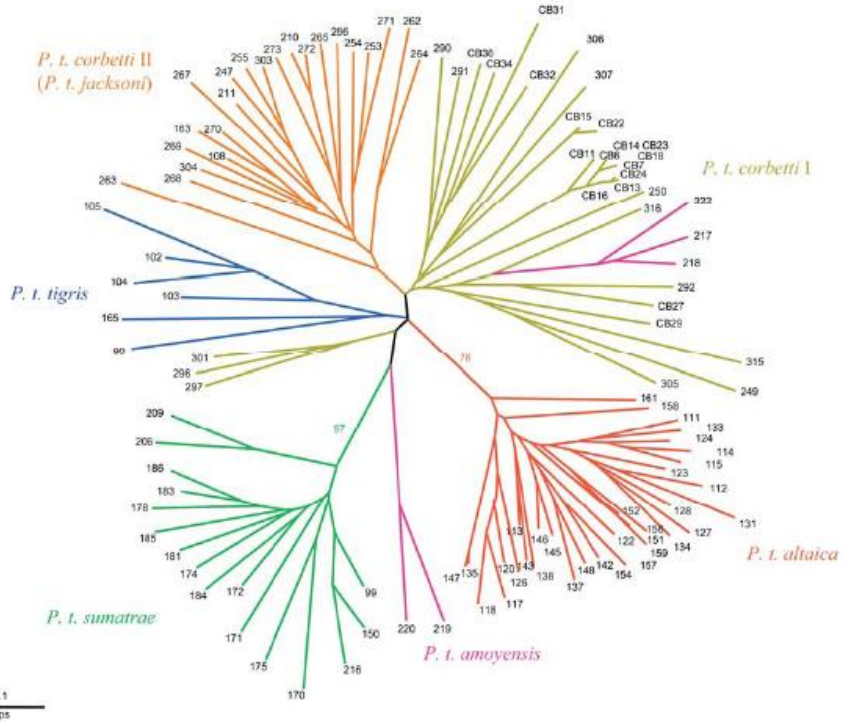
Table 1
The variations in the DNA sequences for 10 bp either side of no. 348 base for some species/subspecies downloaded

	Name	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358
Tiger	*P. t. altaica*	G	A	A	A	T	A	T	C	G	G	G	A	T	T	G	T	G	C	T	A	T
	P. t. corbetti	G	A	A	A	T	A	T	C	G	G	G	A	T	T	G	T	G	C	T	A	T
	P. t. sumatrae	G	A	A	A	T	A	T	C	G	G	G	A	T	T	G	T	G	C	T	A	T
	P. t. tigris	G	A	A	A	C	A	T	C	G	G	G	A	T	T	G	T	G	C	T	A	T
Non-tiger	*P. leo*	G	A	A	A	C	A	T	T	G	G	A	A	T	T	G	T	G	T	T	G	T
	P. b. chinensis	G	A	A	A	C	A	T	T	G	G	A	A	T	C	A	T	A	C	T	G	C
	N. nebulosa	G	A	A	A	C	A	T	T	G	G	A	A	T	C	G	T	A	T	T	A	C
	F. catus	G	A	A	A	C	A	T	T	G	G	A	A	T	C	A	T	A	C	T	A	T
	C. e. hispanicus	G	A	A	A	C	A	T	C	G	G	A	G	T	A	G	T	T	C	T	T	C
	A. a. pfitzmayeri	G	A	A	A	C	A	T	C	G	G	A	G	T	G	A	T	C	C	T	T	C
	O. aries	G	A	A	A	C	A	T	C	G	G	A	G	T	A	A	T	C	C	T	C	C
	C. hircus	G	A	A	A	C	A	T	T	G	G	A	G	T	A	A	T	C	C	T	C	C

Wan, Q. H., & Fang, S. G. (2003). Application of species-specific polymerase chain reaction in the forensic identification of tiger species. *Forensic Science International*, *131*(1), 75-78.

Sub-species identification-Amur tiger





To investigate the species' evolutionary history and to establish objective methods for subspecies recognition, voucher specimens of blood, skin, hair, and/or skin
biopsies from 134 tigers with verified geographic origins or heritage across the whole distribution range were examined for three molecular markers:

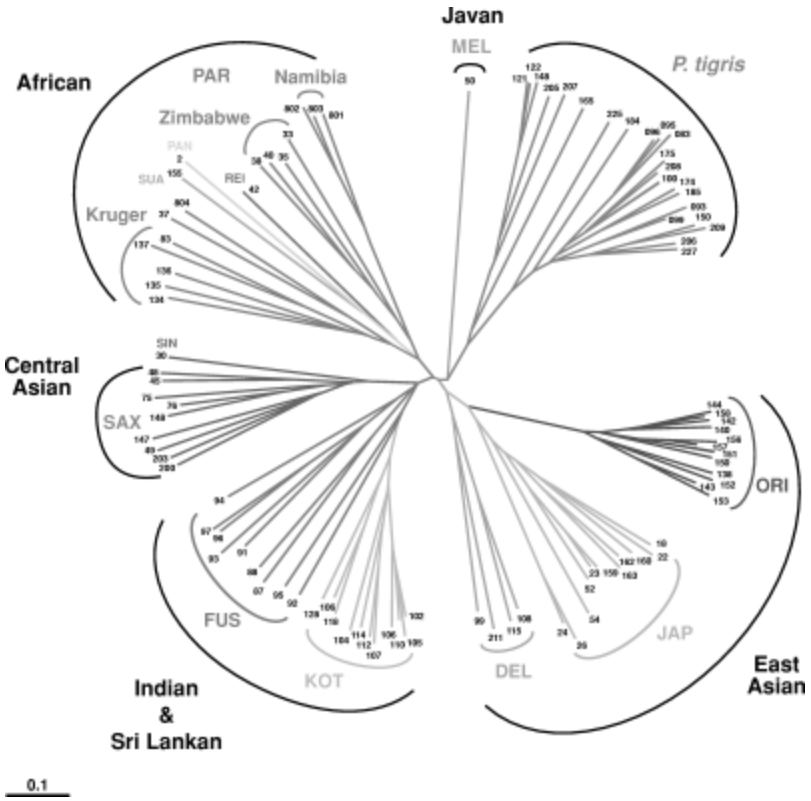
(1) 4 kb of mtDNA sequence
(2) MHC DRB gene;
(3) 30 Microsatellite loci

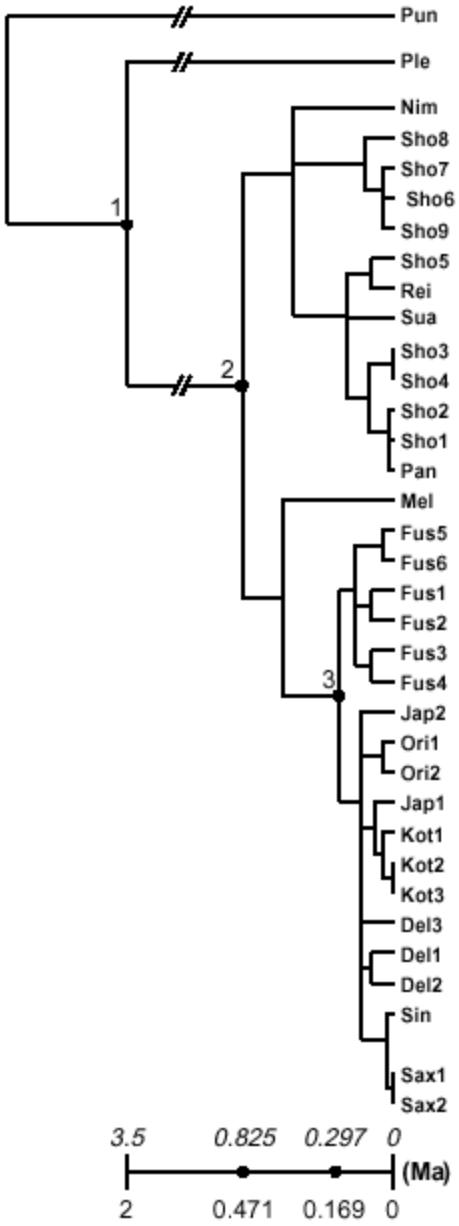
Population genetic structure suggested the recognition of the sixth subspecies Malayan tiger P. t. jacksoni

Luo, S. J., Kim, J. H., Johnson, W. E., Walt, J. V. D., Martenson, J., & Karanth, U. K. (2004). Phylogeography and genetic ancestry of tigers (Panthera tigris). *PLoS biology*, *2*(12), 2275-2293.

Sub-species identification-Amur leopard

Phylogenetic analysis of mitochondrial DNA sequences (727 bp of *NADH5* and control region) and 25 polymorphic microsatellite loci conducted byOlga Uphyrkina *et al* confirmed the sub-species of Amur leopard and revealed its taxonomic status.





2.2 How many are there ?

Individual identification-Amur tiger

TARO SUGIMOTO et al. identify we identified 12 tigers (5 males and 7 females) using 10 microsatellite from more than 100 noninvasive genetic samples, such as feces, hairs, and saliva, collected from southwest Primorye Krai during 4 winters (2000–2001, 2001–2002, 2002–2003, and 2004–2005).

10 microsatellite loci (6HDZ089, FCA043, FCA077, FCA090, FCA094, FCA105, FCA123, FCA161, FCA224, and FCA441) were selected from 18 loci developed by Williamson et al.(2002) and 25 loci used by Uphyrkina et al. (2001)

Individual	Total no. observations	Winters observed			
		2000–2001 (n = 6)	2001–2002 (n = 14)	2002–2003 (n = 42)	2004–2005 (n = 17)
MT1	12	1	1	7	3
MT2	2	1	0	1	0
MT3	10	0	4	6	0
MT4	1	0	0	1	0
MT5	5	0	0	0	5
FT1	1	1	0	0	0
FT2	1	1	0	0	0
FT3	6	0	1	3	2
FT4	3	0	0	1	2
FT5	1	0	0	1	0
FT6	2	0	0	2	0
FT7	2	0	0	2	0
Total (n feces/n hairs)	46 (40/6)	4 (4/0)	6 (5/1)	24 (23/1)	12 (8/4)

Sugimoto, T., Nagata, J., Aramilev, V. V., & McCullough, D. R. (2012). Population size estimation of Amur tigers in Russian Far East using noninvasive genetic samples. *Journal of mammalogy*, *93*(1), 93-101.

Individual identification-Amur tiger in China

Eleven scat samples were collected within and adjacent to the reserve during tiger monitoring surveys, of which, nine scat samples were identified as tiger using six mitochondrial DNA primer sets.

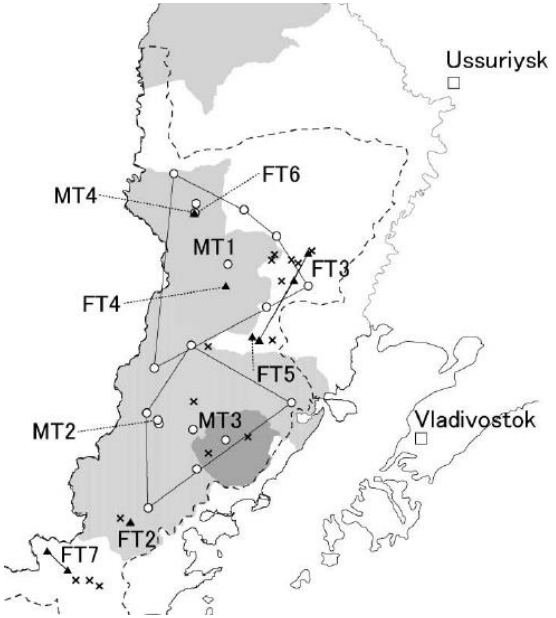
9 microsatellite loci were used to discern a total of five individuals (4 males and 1 female), each sample underwent at least four independent PCR reactions per locus

FIG. 1 Location of Hunchun Nature Reserve, showing sampling localities where tiger scats were and were not identified. Some sampling localities were outside the Reserve because there was anecdotal evidence of tigers in these areas. The black circle indicates the nearest tiger population in Russia (Russello et al., 2004) and the inset the location of the main map on the China–Russia border. The three shades of grey indicate different zones within the Reserve.

Caragiulo, A., Kang, Y., Rabinowitz, S., Dias-Freedman, I., Loss, S., Zhou, X. W., ... & Amato, G. (2015). Presence of the Endangered Amur tiger Panthera tigris altaica in Jilin Province, China, detected using non-invasive genetic techniques. *Oryx*, 1-4.

Population size -Amur tiger

TARO SUGIMOTO et al. identify we identified 12 tigers from more than 100 noninvasive genetic samples collected from southwest Primorye Krai during 4 winters, and population size estimated from the 2002–2003 samples was 12 (95% confidence interval 5 9–19)
In addition, Of the 3 types of noninvasive genetic samples we collected, feces were the most useful samples in tiger population size estimation.



Effective population size -Amur tiger

P. HENRY et al. sampled 95 individuals collected throughout their native range in Russia and estimates of effective population size (Ne = 27–35) and Ne/N ratio (0.07– 0.054), which is quite low.

MOLECULAR ECOLOGY
Molecular Ecology (2009) 18, 3173–3184 doi: 10.1111/j1365-294X.2009.04266.x

In situ population structure and *ex situ* representation of the endangered Amur tiger

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Abstract

The Amur tiger (*Panthera tigris altaica*) is a critically endangered felid that suffered a severe demographic contraction in the 1940s. In this study, we sampled 95 individuals collected throughout their native range to investigate questions relative to population genetic structure and demographic history. Additionally, we sampled targeted individuals from the North American *ex situ* population to assess the genetic representation found in captivity. Population genetic and Bayesian structure analyses clearly identified two populations separated by a development corridor in Russia. Despite their well-documented 20th century decline, we failed to find evidence of a recent population bottleneck, although genetic signatures of a historical contraction were detected. This disparity in signal may be due to several reasons, including historical paucity in population genetic variation associated with postglacial colonization and potential gene flow from a now extirpated Chinese population. Despite conflicting signatures of a bottleneck, our estimates of effective population size (N_e = 27–35) and N_e/N ratio (0.07–0.054) were substantially lower than the only other values reported for a wild tiger population. Lastly, the extent and distribution of genetic variation in captive and wild populations were similar, yet gene variants persisted *ex situ* that were lost *in situ*. Overall, our results indicate the need to secure ecological connectivity between the two Russian populations to minimize loss of genetic diversity and overall susceptibility to stochastic events, and support a previous study suggesting that the captive population may be a reservoir of gene variants lost *in situ*.

Keywords: bottleneck, captive, conservation genetic, microsatellite, *Panthera tigris altaica*, population genetic structure

Received 17 November 2008; *revision received* 30 April 2009; *accepted* 5 May 2009

2.3 How are they ?

Sex ratio-Amur tiger and leopard

For sex identification, Sugimoto et al designed primers specific to both tiger and leopard
ZFX-PF (5¢TACCGAGCGATATAGCTCCAG-3¢) /ZFX-PR (5¢-GTGTTCCTACGTTAAGCTATTG-3¢) for X chromosome and
DBY7-PF (5¢-CTCATGAAGCCCTATTTTTGGTTG-3¢) /DBY7-PR (5¢-ACGGCGTCCGTATCTTCCA-3¢) for Y
their fragment sizes were 205 and 156 bp, respectively.

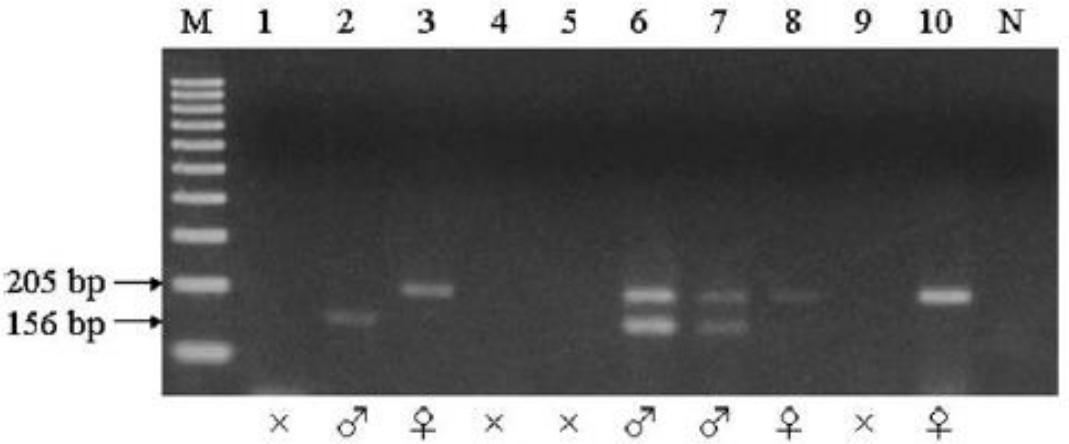


Figure 2. Examples for sex identification from faecal samples. DBY fragments are present in lanes 2, 6 and 7, and samples were from males. Zfx fragments are present but DBY fragments are absent in lanes 3, 8 and 10, and samples were from females. DNA amplification is unsuccessful in lanes 1, 4, 5 and 9.

Sugimoto, T., Nagata, J., Aramilev, V. V., Belozor, A., Higashi, S., & McCullough, D. R. (2006). Species and sex identification from faecal samples of sympatric carnivores, Amur leopard and Siberian tiger, in the Russian Far East. *Conservation Genetics*, *7*(5), 799-802.

Genetic diversity -Amur tiger and leopard

Table 3 Comparison of genetic diversity between different felid populations

物种 Species	研究区域 Study area	等位基因数 Na	期望杂合度 He
东北虎 *Panthera tigris altaica*	吉林珲春自然保护区 Hunchun Nature Reserve, Jilin Province	2.64	0.371
	俄罗斯远东滨海边境区域 Primorye Krai, Russian Far East	3.20	0.580
	扬州市茱萸湾动物园 Zhuyuwan Zoo, Yangzhou	5.53	0.750
	内蒙古赛罕乌拉自然保护区 Saihanwula Nature Reserve, Inner Mongolia	2.88	0.547
欧亚猞猁 *Lynx lynx*	波兰和白俄罗斯边界地区 Polish and Belarussian border	3.97	0.500
	挪威和瑞典的 Scandinavia 半岛 Scandinavian Peninsula of Sweden and Norway	4.70	0.510
孟加拉虎 *Panthera tigris tigris*	印度 Nehru 动物园 Nehru Zoological Park, India	6.40	0.740
雪豹 *Panthera uncia*	尼泊尔东部地区 East of Nepal	3.83	0.579

Zhou et al. found that genetic diversity of Amur tiger is relatively lower than other felids. And the parameter of tigers in China is slightly lower than in adjacent Russia Fareast.

The application of extracting DNA from noninvasive samples in feline species，Zhou et al., 2015

Olga Uphyrkina et al. noted that Recent demographic reductions likely have led to genetic impoverishment in *P. p. orientalis*.

Uphyrkina, O., Johnson, W. E., Quigley, H., Miquelle, D., Marker, L., Bush, M., & O'Brien, S. J. (2001). Phylogenetics, genome diversity and origin of modern leopard, Panthera pardus. *Molecular ecology*, *10*(11), 2617-2633.

Table 5. Genetic variation across mtDNA gene segments (*NADH-5*, 611 bp, and Control Region, 116 bp) and 25 microsatellite loci in seven revised leopard subspecies

	mtDNA					Microsatellites					
Subspecies	Number leopards mtDNA/ μsat	Number variable sites	Mean number pairwise differences (SE)	$\pi \times 10^2$ (SE)	% Polymorphic loci	Average H_E (SE)	Average number alleles/locus	% Specific alleles	Average range repeat/locus	Microsatellite variance	Maximum range
Panthera pardus	69/75	50	8.67 (4.40)	1.21 (0.62)	100	0.793 (0.073)	11.08	—	12.64	7.11	17
P. p. pardus (I + II)	15/17	21	8.77 (4.29)	1.22 (0.67)	100	0.803 (0.076)	8.52	29.1	9.72	7.28	15
PAR I	**11/13**	**14**	**4.78 (2.53)**	**0.67 (0.40)**	**100**	**0.795 (0.099)**	**8.36**	**20.1**	**10.28**	**7.59**	**13**
PAR II	**4/4**	**3**	**3.75 (2.38)**	**0.52 (0.39)**	**100**	**0.675 (0.083)**	**4.08**	**3.92**	**6.00**	**5.24**	**9**
P. p. saxicolor	8/10	2	0.50 (0.47)	0.07 (0.07)	100	0.616 (0.083)	4.24	2.83	5.12	4.28	7
P. p. fusca	9/9	8	2.61 (1.54)	0.36 (0.24)	100	0.696 (0.144)	5.52	5.80	6.2	5.38	9
P. p. kotiya	10/11	2	0.56 (0.50)	0.08 (0.08)	96	0.485 (0.202)	3.52	5.68	4.58	4.25	7
P. p. delacouri	3/4	5	3.41 (2.37)	0.48 (0.41)	100	0.674 (0.126)	4.20	5.71	5.56	5.70	6
P. p. japonensis	9/11	1	0.95 (0.71)	0.21 (0.15)	100	0.549 (0.171)	3.76	3.19	4.44	2.70	7
P. p. orientalis	12/12	1	0.17 (0.24)	0.02 (0.04)	92	0.356 (0.222)	2.60	3.07	2.84	1.71	4

Genetic structure -Amur tiger

P. HENRY et al. sampled 95 individuals collected throughout their native range in Russia and Bayesian structure analyses clearly identified two populations separated by a development corridor, and recommend to secure ecological connectivity between the two Russian populations to minimize loss of genetic diversity.

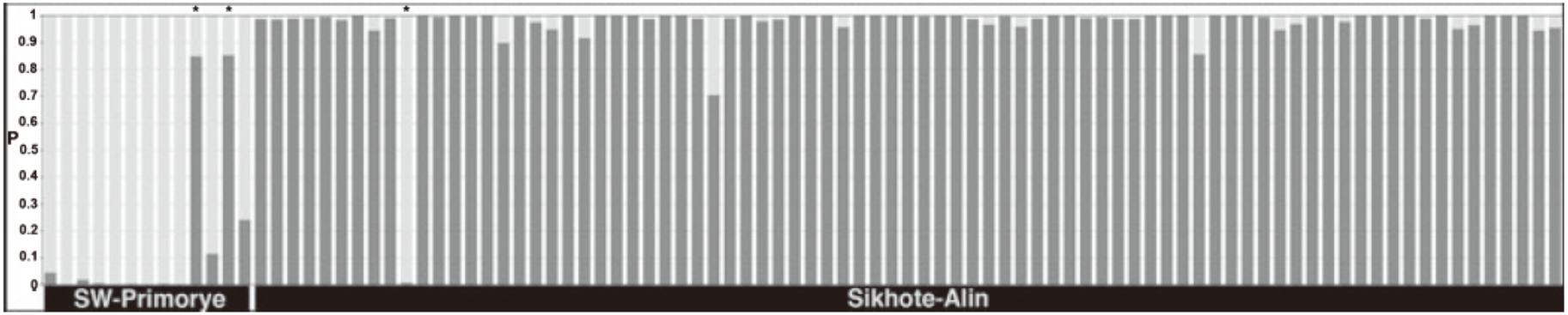


Fig. 2 Plot indicating the genetic composition of wild Amur tiger populations, expressed in terms of assignment probability (*P*) according to the Bayesian method of Francois *et al.* (2006). The relative contributions of each of the two genetic partitions recovered from the data are indicated by colour for each individual (column) in each sampled population. The Southwest Primorye cluster is represented in light grey while Sikhote-Alin is in dark grey. The three potential migrant individuals are identified with an asterisk above their corresponding columns.

Henry, P., Miquelle, D., Sugimoto, T., McCullough, D. R., Caccone, A., & Russello, M. A. (2009). In situ population structure and ex situ representation of the endangered Amur tiger. *Molecular Ecology*, *18*(15), 3173-3184.

Health condition- MHC variability

- The major histocompatibility complex (MHC) controls a major part of the immune system in mammals and the variation of MHC genes can be a indicator of wildlife health condition.

- The study of SL Hendrickson *et al.* demonstrated that of the three tiger subspecies (Bengal, *Panthera tigris tigris*; Siberian, *P. t. altaica*; Sumatran,*P. t. sumatrae*), because of largest sample of both captive and wild tigers, Amur tigers provided the best estimates of variability and captive tigers were less so than the wild samples.
- We have to point out that it is still hard to do MHC variability analysis because of the complex of MHC structure.

Table 3. Mean average percentage difference among individuals (MAPD) and its standard error (SEM), both calculated from unweighted enzyme APD values, at the MHC class I complex for several mammalian species

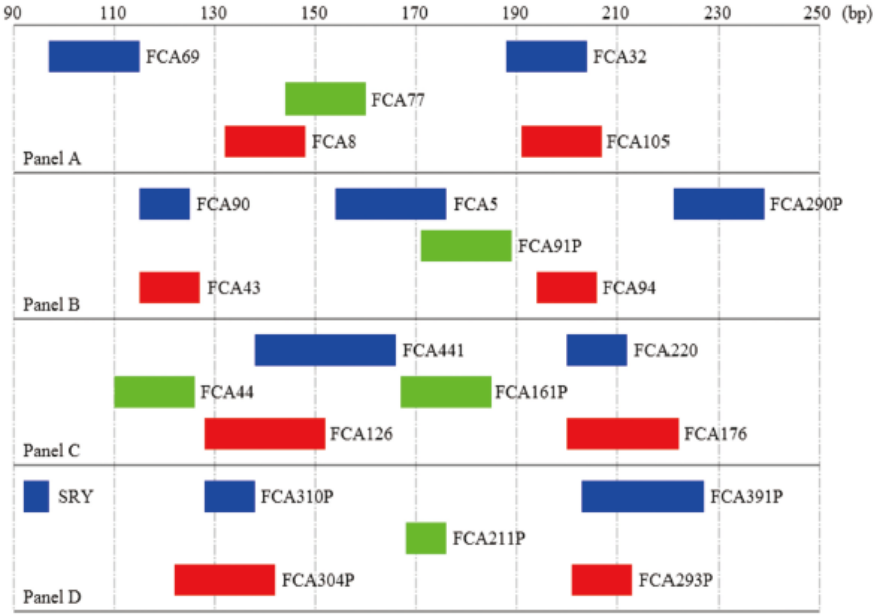
Population	MAPD	SEM	Reference
Wild Siberian tiger	9.06	4.53	This study
Wild Sumatran tiger	4.79	2.41	This study
Wild Bengal tiger	1.28	1.28	This study
Captive Siberian tiger	5.26	1.90	This study
Captive Sumatran tiger	6.71	1.71	This study
Captive Bengal tiger	8.43	3.01	This study
Serengeti lion	8.8	1.3	Yuhki & O'Brien (1989)
Ngorongoro lion	5.8	1.3	Yuhki & O'Brien (1989)
Gir Forest lion	0	0	Yuhki & O'Brien (1989)
East African cheetah	2.2	0.8	Yuhki & O'Brien (1989)
South African cheetah	2.1	0.8	Yuhki & O'Brien (1989)
House cats	10.2	1.1	Yuhki & O'Brien (1989)
Humans	9.8	1.7	Yuhki & O'Brien (1989)

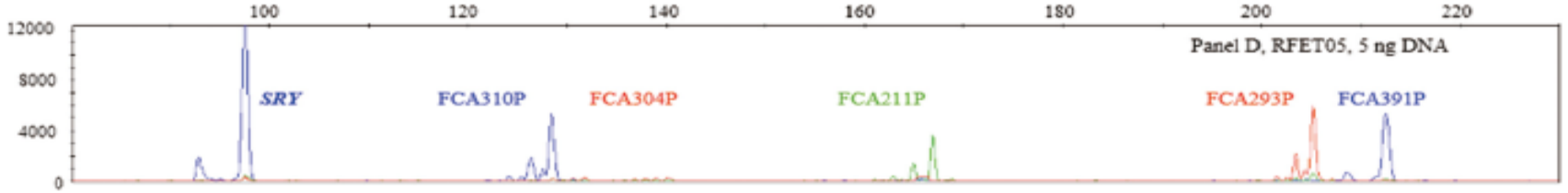
Hendrickson, S. L., Mayer, G. C., Wallen, E. P., & Quigley, K. (2000). Genetic variability and geographic structure of three subspecies of tigers (Panthera tigris) based on MHC class I variation. *Animal Conservation*, *3*(2), 135-143.

2.4 What is good --microsatellite markers for multiplex PCR

Zou *et al.* developed a multiplex genotyping system "tigrisPlex" composed of STR loci and a gender-identifying *SRY gene, amplified in 4 reactions using as little as 1 ng of template DNA.*

They applied "tigrisPlex" to 12 confiscated specimens from Russia and identified 6 individuals (3 females and 3 males)
and further they applied a complete Bayesian clustering method implemented in STRUCTURE *to calculate the probability (q) of a tiger with unknown identity belonging to each of the 5 putative subspecies using the voucher tiger subspecies samples (n = 113 [Luo et al. 2004]) as a reference* and all designated as Amur tigers (*Panthera tigris altaica) with high confidence.*





ZOU, Z. T., UPHYRKINA, O., FOMENKO, P., & LUO, S. J. (2015). The Development and Application of a Multiplex STR System for Identifying Subspecies, Individuals and Sex in Tigers. *Integrative zoology*.

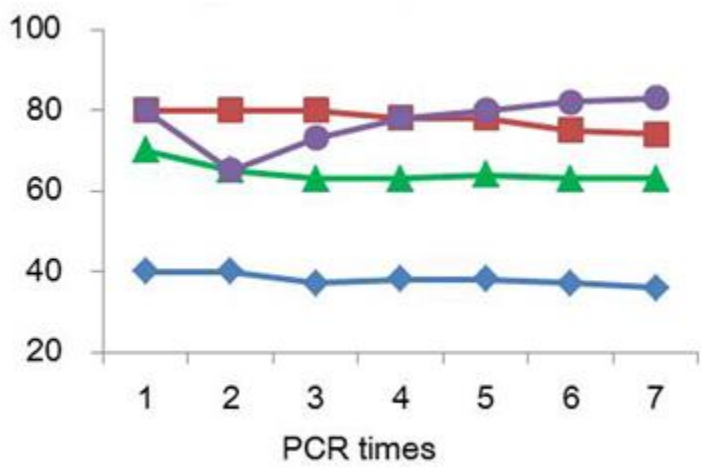
Part 3 Will ‘good’ be ‘perfect’ ?

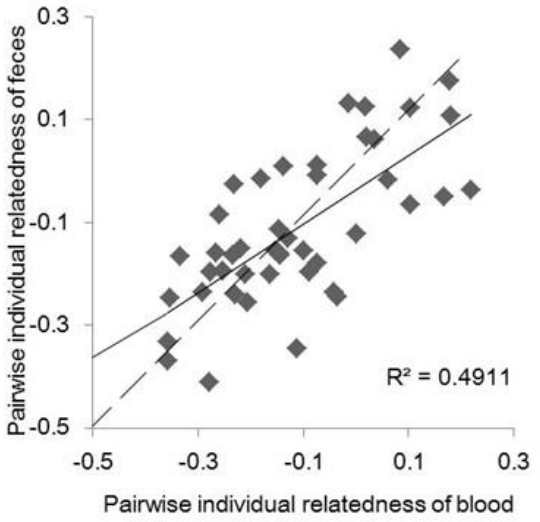
In our recent piot study, we assessed genotyping risks across 12 microsatellite loci that were commonly used in tiger studies (**good loci**) using blood and fecal DNA from captive Amur tigers *Panthera tigris altaica.*

We repeated as many as 7~10 PCR times for some loci, but the genotyping results of some fecal DNA amplifications were still different from those of blood of the same individual.

YES ! Genotyping errors always existed .

So, we strongly recommend that when using scats as DNA resources, pilot study of genotyping error rate must be conducted to correct the final result.





Part 4 Recommendations

1. As discussed in Part 3, even ‘good’ loci may face great genotyping risks, we advocate to build a standardized genetic marker system that is suitable for non-invasive samples, such as scats and hair.

2. Multiplex PCR should be promoted to simplify our experimental procedure and improve the reliability of our genetic analysis results.

3. Establish a genotyping database with the standardized system which contains genetic data of Amur tiger and leopard in the whole Northeast China and Russia Fareast areas by all the researchers. So that we can share and exchange our information and promote to develop make more comprehensive conservation and management implications.

Part 5 Our methods

- ✓ Genetic samples preservation
- ✓ DNA extraction
- ✓ Genetic markers selection
- ✓ PCR and genotyping

5.1 Genetic samples preservation

- We collected blood, urine and scats samples with ziplock bags and sterile gloves, and reserved the samples outdoors in the winter and portable ice boxes in other seasons.

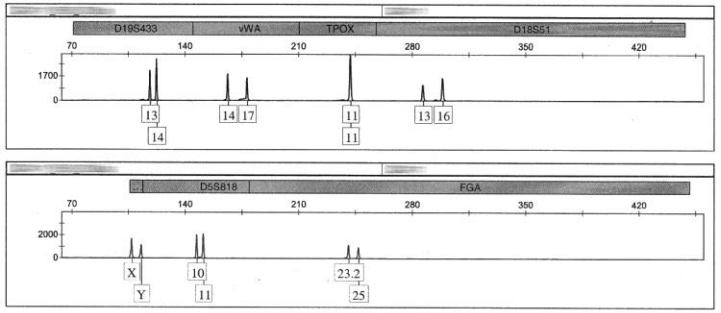
- Preserve in Ultra-low Temperature Freezer (- 80 ℃) until DNA extraction.

5.2 DNA extraction

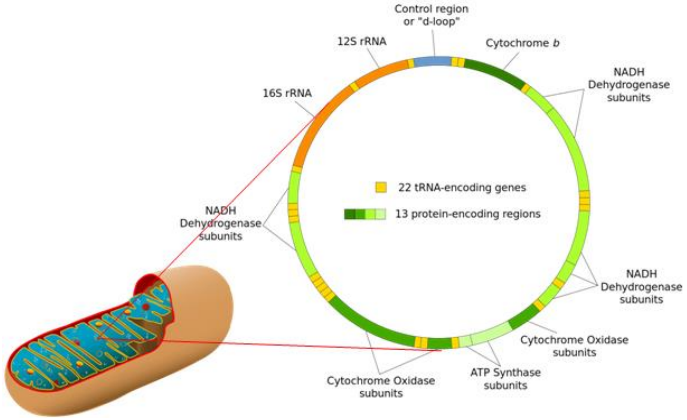
Sample type	Extraction method	Remarks
Blood	QIAGEN blood & tissue kit (QIAGEN, Inc.)	
Tissue	Phenol/chloroform method & DNeasy Tissue Kit (Qiagen, Valencia, CA)	
Scat	QIAamp DNA Stool Mini Kit (50) (Qiagen)	With slight modifications, peer off about 5 g of feces and preserved in absolute ethanol for 24 h before extraction according to operation manual.
Urine	Phenol/chloroform method	
Hair	QIAamp DNA Stool Mini Kit (50) (Qiagen)	At least 3 hair with follicle are needed
Saliva	QIAamp DNA mini kit (Qiagen, Inc.)	

5.3 Genetic markers selection

- Microsatellite loci

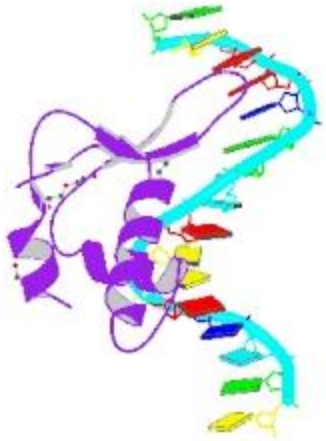


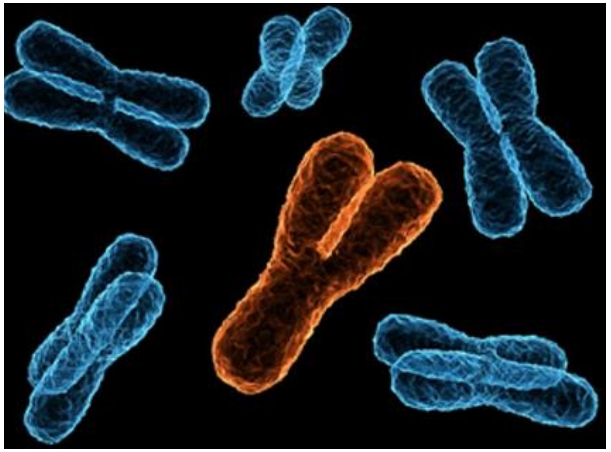
- mtDNA fragment



- SRY gene

- MHC





Microsatellite loci

We selected 12 microsatellite loci for Amur tiger and leopard from 35 loci presented in previous studies.

	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

Samrat Mondol et al., 2009; Jyotsna Bhagavatula and Lalji Singh, 2006; T. Sugimoto et al., 2014

mtDNA fragment

1. Leopard soecies-specific primer: Ppo-CbF (5¢-GTAAATTATGGCTGAATTATCCGG- 3¢) /Ppo-CbR (5¢- CATAACCGTGAACAATAATACGAC-3¢); **(156bp)**
2. Tiger species-specific primer, Pta-CbF (5¢-TTTGGCT CCTTACTAGGGGTG-3¢) /Pta-CbR (5¢-CCGT AAACAATAGCACAATCCCGATA-3¢) **(217bp)**

Table 1. PCR Primers Specific for Cytoplasmic Mitochondrial DNA Sequences

Primer ID	Mitochondrial Segments	Forward[a]	Reverse[a]	Size(bp)
C53F1/T598R	*ND5*	CCCAGATCCCTATATTAACCAGT	TATATCATTTTGTGTGAGGGCAC	546
C708F/T1300R	*ND5*	CCTTGTCTTCCTGCATATCTG	CCATTGGAAAGTACCCGAGGAGGT	593
C1494F/T1936R	*ND6*	TCTCCTTCATAATCACCCTGA	TGGCTGGTGGTGTTGGTTGCGG	443
C2339F/T2893R	*CytB*	TTGCCGCGACGTAAACCACG	GTTGGCGGGGATGTAGTTATC	555
CR-UPF/CR-R2B	*CR*	TCAAAGCTTACACCAGTCTTGTAAACC	CGTGTTGTGTGTTCTGTAT	250
C4979F/T5424R[b]	*12S*	GCACTGAAAATGCCTAGATGAGT	CCAGTTTGGGTCTTAGCTATCG	446
C-12S-F/N/C-12S-R	*12S*	AAAGCCACAGTTAACGTAA	TACGACTTGTCTCCTCTTGTGG	577
T7812F/C8294R[b]	*ND1*	CGTCGTAGGACCATACGGCC	CTCAGTCTCCTTCTGTTAAAT	483
C8276F/T8620R	*ND1*	CGAAGCGAGCTCCATTTGATTTA	GTGGAATGCTTGCTGTAATGATGGG	345
T8942F/C9384R	*ND2*	CTTATAGTCTGAATCGGCTTCG	AGCTATGATTTTTCGTACCT	443
C9366F/T9882R	*ND2*	GGGGAGTTAACCAAACCGAG	CAAGGACGGATAGTATTGGTG	517
C10525F/T11013R[b]	*COI*	GGAGGATTCGGAAACTGGCGA	CCAGAAGTCTATATCTTAATCCCG	489
C11020F/T11428R	*COI*	CCAGAAGTCTATATCTTAATCCCG	GCTCCTATTGACAAGACGTAGTGGA	409
T11988F/C12414[b]	*COII*	GGCATACCCCTTCCAACTAGGT	TGCACACTTCTATTGCTAGT	427
C12618F/T12920R[b]	*ATP8*	TTGTCCATGAACTAGTCCCATCAT	GGAAACAGCTATGACCGGCG	303

Luo, S. J., Kim, J. H., Johnson, W. E., Walt, J. V. D., Martenson, J., & Karanth, U. K. (2004). Phylogeography and genetic ancestry of tigers (Panthera tigris). *PLoS biology*, *2*(12), 2275-2293.

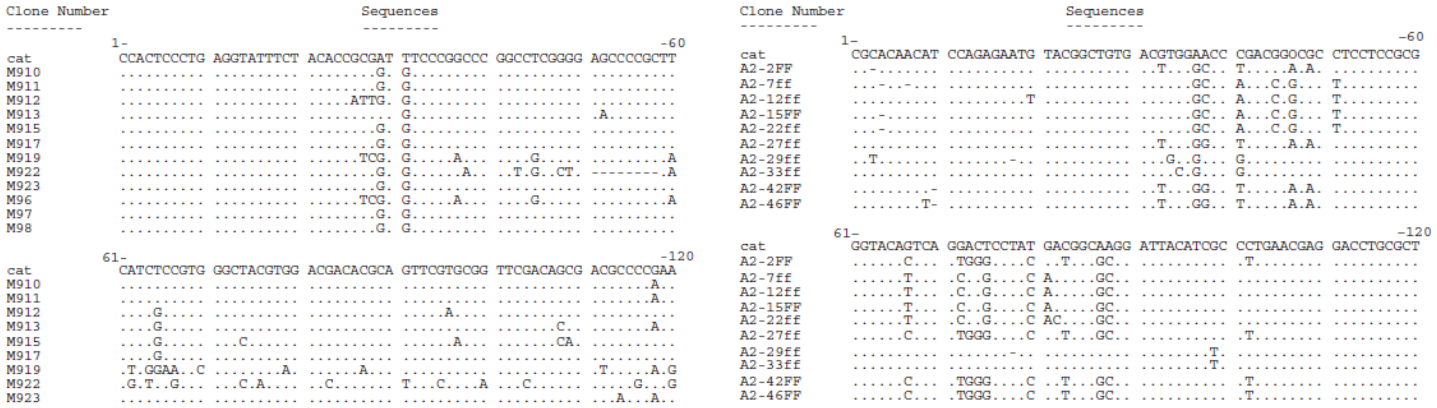
Sex determination gene

For sex identification, we used primers specific to both tiger and leopard
ZFX-PF (5¢TACCGAGCGATATAGCTCCAG-3¢) /ZFX-PR (5¢-GTGTTCCTACGTTAAGCTATTG-3¢) for X chromosome and
DBY7-PF (5¢-CTCATGAAGCCCTATTTTTGGTTG-3¢) /DBY7-PR (5¢-ACGGCGTCCGTATCTTCCA-3¢) for Y
their fragment sizes were 205 and 156 bp, respectively.

MHC gene

MHC class I :
Catalpha1F : 5’-CCACTCCCTGAGGTATTTCTACACC-3’;
Catalpha2R: 5’-TGTCCAGGTATTTGGCGAGC-3’

MHC II DRB:
DRB219: 5’-CCACACAGCGTTTCYTG-3’;
DRB61A:5’-CCGCTGCACTGTGAAGCT-3’



Sachdev, M., Sankaranarayanan, R., Reddanna, P., Thangaraj, K., & Singh, L. (2005). Major histocompatibility complex class I polymorphism in Asiatic lions. *Tissue antigens*, *66*(1), 9-18.

KUWAHARA, Y., KITOH, K., KOBAYASHI, R., IWATA, J., OHNE, R., HOSOKAWA-KANAI, T., ... & SASAKI, Y. (2000). Genotyping of feline MHC (FLA) class II DRB by PCR-RFLP method using group-specific primers.*Journal of Veterinary Medical Science*, *62*(12), 1283-1289.

5.4 PCR and genotyping

- ✓ **Reaction volume:** PCRs were performed in a reaction volume of 20 μL, containing approximately 30 ng of genomic DNA, 10 μ l of 2× PCR Buffer for KOD FX Neo, 0.75 μL of each primer (Invitrogen, Paisly, UK), 2 mM dNTPs, and 0.3 μ l of KOD FX Neo (1 U/μl) (TOYOBO, Japan)
- ✓ **Reaction system:** Cycling was performed using the following conditions: An initial denaturation step for 5 mins at 98° C was followed by 35 cycles of 15 s of denaturation at 95° C, 30 s of annealing at 58° C, and 20 s of extension at 68° C, followed by a final extension step for 10 min at 68° C.
- ✓ Genotyping: The lengths of microsatellite fragments were determined in an ABI 3130 automatic genetic analyzer using the Liz 500 standard and the GeneMapper 4.0 software (Applied Biosystems, United States). To increase data significance, PCR with DNA from feces was performed at least 3 times for heterozygote and 5 times for homozygote.


Thank you for your attention !