The analysis of genetic data of the Amur tiger and Amur leopard samples from the field

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Outline:

1.Non-invasive samples collection;

2.How to treat with the samples;

3. Analysis results of the samples;

4. Summary.

Questions:

1 Of all the samples in field,which belong to Amur tiger and which belong to Amur leopard?

2. How many Amur Tigers and Amur leopards exist in China now?

3. How about genders of Amur tiger and population based on genectic analysis?

1.Non-invasive samples collection

① Number of samples

The 245 suspected samples of Amur tigers and leopards were colleted since 2010 across the range;

② Sample types

Including 169 fecal samples, 27 hair samples ,14 blood samples , 22 urine samples and 13 saliva samples.

2.How to treat with the samples

- **DNA extraction**

  Blood samples:

  DNA was extracted through the standard phenol: chloroform method (Sambrook et al., 1989).

Fecal samples:

All the DNAs of fecal samples were extracted through the modified QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) as described in Zhang et al. (2009).

2. How to treat with the samples

- **Improvements of DNA extraction methods of the fece samples**

1) Make about 5g of feces (peeled from the surface of each fecal pellet)deactivated in 100% ethanol with volume ratio at room temperature for 12h.

2) Vortex the feces and ethanol at 2,200 rpm for 3 min.

3) Filtrate the mixture through a piece of sterile gauze.

4) Collecte the filtrate and centrifuge it at 3,500 rpm for 15 min.

5) Transfer the pellets at the bottom to a new tube for extraction.

6) The following can be done as the instructions.

2. How to treat with the samples

●Mitochondrial DNA Amplification

1) Amur tiger species identification(annealing60℃)

Pta-CbF（5-TTTGGCTCCTTACTAGGGGTG-3）

Pta-CbR（5-CCGTAAACAATAGCACAATCCCGATA-3）

2) Amur leopard species identification

Ppo-CbF (5'-GTAAATTATGGCTGAATTATCCGG-3')

Ppo-CbR (5'-CATAACCGTGAACAATAATACGAC-3')

2. How to treat with the samples

- **Species identification**

① 69 fecal samples,5 urine samples, 14 blood samples and 4 hair samples were confirmed to be Amur tigers;

② 55 stool samples and 4 urine samples were Amur leopards;

③ 13 samples will be tested.

2. How to treat with the samples

- Characterizations and conditions of polymerase chain reaction for 8 microsatellite loci

微卫星位点 Microsatellite locus	引物（5'-3'） Primers（5'-3'）	重复单元 Repeat motif	退火温度 Annealing temperature (°C)	PCR产物大小范围 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
Fca304	TCATTGGCTACCACAAAGTAGG CTGCATGCCATTGGGTAAC	$(GT)_{11}(GG)_3(GT)_8$	58	120-134
Fca043	GAGCCACCCTAGCACATATACC AGACGGGATTGCATGAAAAG		58	116-130
FCA391	GCCTTCTAACTTCCTTGCAGA TTTAGGTAGCCCATTTTCATCA	$(ATGG)_{15}(GATA)_{11}(TAGA)_2TGA(TAGA)_2$	55	190-230
Fca152	TTTAGTCAGCTTAGGCTTCCA CTTCCCAGCTTCCAGAATTG	$(AC)_{21}$	58	129-147
FCA441	ATCGGTAGGTAGGTAGATATAG GCTTGCTTCAAAATTTTCAC	$(ATAG)_8(GTAG)_3(ATAG)_2AG(ATAG)_2$	58	130-168

2. How to treat with the samples

●PCR conditions

① PCR was set up 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 dN; TP (TOYOBO), 0.2 uM each of forward and reverse primer, 0.4 U units of KOD FX Neo DNA polymerase (TOYOBO) and ~80 ng of total DNA.

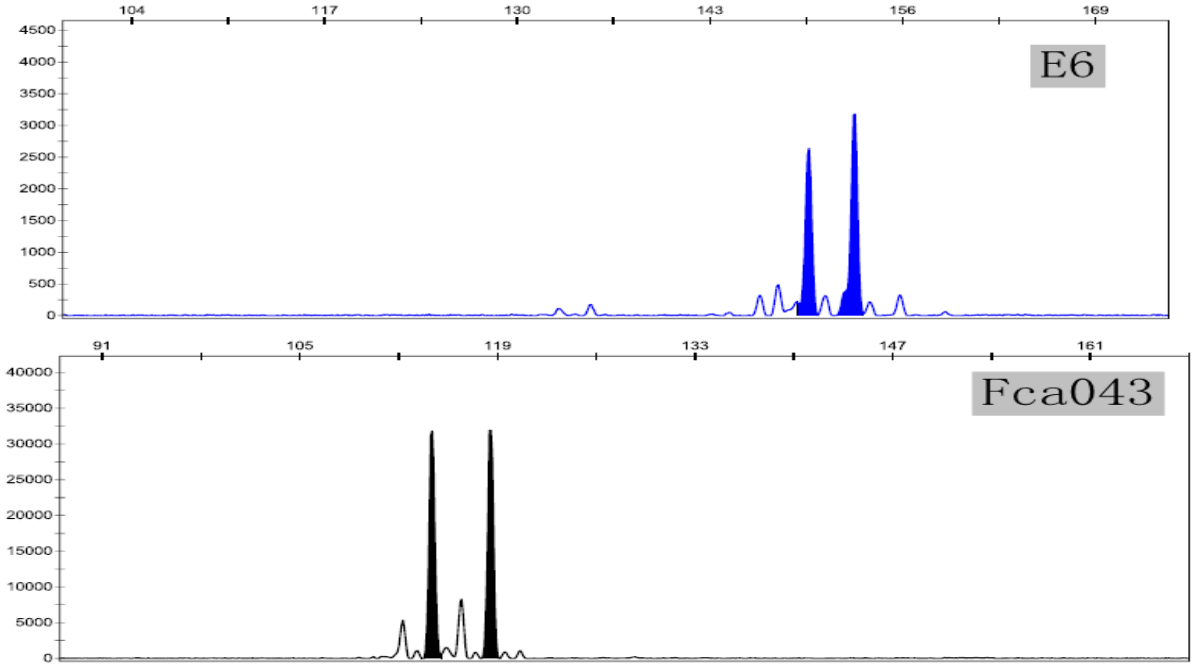
②PCR amplification was performed on a Model 9700 Thermocycler (Perkin-Elmer) using the following program: 1 cycle of 2 min at 94° C, 35 cycles of 98° C for 20 s, annealing temperature (55–62° C, Table 1) for 30 s, 68° C for 20 s, and 1 cycle of 68° C for 20 min.

2. How to treat with the samples

●Amplification and analysis methods:

1) Ten microsatellite loci were selected for the amplification of field samples by using the fluorescence labeling technique of field sample in this experiment.

2) Analysis the size of the amplified products by using 3100DNA sequencing



2. How to treat with the samples

- Multi-tube PCR-based standard for genotyping

Repeat every sample 3~5 times , when more than twice same cases occur, we regard it as correct.

		first		second		third		fourth		fifth		result	
8	tiger	148.3	150.49	148.32	150.49	148.32	150.59					148	150
9	tiger	148.3	150.49	133.22	148.21	145.99	148.32	148.33	150.58	148.23	150.49	148	150

A	B	C	D	E	F	G	H	I	J	K	L	M	N
		first		second		third		fourth		fifth		result	
102	lepord	179.96		126.53	154.13	156.27	158.1	150.67	154.19	150.2	152.15	0	0
106	lepord	156.25	158.22	156.33	162.01	153.85		154.3	156.31	154.17	156.07	0	0

2. How to treat with the samples

●D10 and FCA391 sites for leopards were discarded due to poor signal.

		D10		FCA391	
20B	lepord	0	0	206	210
21B	lepord	156	160	0	0
24B	lepord	156	158	210	214
28B	lepord	0	0	210	210
30B	lepord	0	0	0	0
33B	lepord	0	0	0	0
34B	lepord	158	160	0	0
35B	lepord	152	162	0	0
36B	lepord	152	162	0	0
63B	lepord	0	0	0	0
64B	lepord	0	0	0	0
65B	lepord	0	0	206	206
66B	lepord	0	0	0	0
67B	lepord	0	0	0	0
68B	lepord	156	158	206	214
69B	lepord	0	0	0	0
70B	lepord	0	0	0	0
71B	lepord	0	0	0	0
73B	lepord	0	0	0	0
74B	lepord	0	0	0	0
75B	lepord	0	0	0	0
76B	lepord	0	0	0	0
77B	lepord	152	162	0	0
79B	lepord	0	0	0	0

3. Analysis results of the samples.

- **Probability of Amur leopard individual identification**

This is the P(ID) result, we can see from the table that the probability of identifing leopard individuals with 8 loci is quite high, reaching 9.564e-08, and even for sibs, the value can be 1.941e-03

locus	biased/loci	unbias./loc.	sibs/locus	Prod(biased)	Prod(unbias)	Prod(sibs)
E6	1.245e-01	1.087e-01	4.214e-01	1.245e-01	1.087e-01	4.214e-01
E21	1.823e-01	1.629e-01	4.764e-01	2.270e-02	1.770e-02	2.008e-01
FCA304	1.006e-01	8.625e-02	3.980e-01	2.282e-03	1.527e-03	7.990e-02
FCA043	3.065e-01	2.957e-01	5.528e-01	6.996e-04	4.514e-04	4.417e-02
FCA152	4.222e-01	3.905e-01	6.765e-01	2.954e-04	1.763e-04	2.989e-02
FCA441	5.793e-02	4.553e-02	3.554e-01	1.711e-05	8.025e-06	1.062e-02
F53	8.698e-02	7.322e-02	3.850e-01	1.488e-06	5.876e-07	4.089e-03
F85	1.816e-01	1.628e-01	4.747e-01	2.703e-07	9.564e-08	1.941e-03

3. Analysis results of the samples.

- Genotypes of 8 microsatellite loci of all samples for leopards.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1		E6		E21		FCA304		FCA043		FCA152		FCA441		F53		F85	
2	21B	150	153	156	162	112	114	115	119	125	125	0	0	0	0	0	0
3	24B	0	0	156	162	0	0	115	119	125	125	140	148	0	0	149	153
4	28B	150	153	0	0	0	0	119	119	125	125	156	156	0	0	153	161
5	36B	0	0	156	166	112	114	115	119	125	125	0	0	126	134	149	153
6	63B	153	153	162	156	112	114	115	119	125	125	140	140	126	134	149	153
7	65B	153	150	0	0	112	114	115	119	125	125	148	148	126	134	149	153
8	66B	150	153	156	162	112	114	115	119	125	125	0	0	126	138	149	153
9	67B	0	0	0	0	0	0	115	119	125	125	140	152	122	122	153	157
10	68B	153	153	156	162	112	114	115	119	125	125	0	0	126	134	149	153
11	69B	0	0	156	158	112	114	115	119	125	125	140	152	122	122	153	157
12	70B	129	150	0	0	0	0	115	119	125	125	132	132	126	134	0	0
13	71B	150	147	154	156	0	0	115	119	125	139	148	148	0	0	153	153
14	74B	153	153	154	156	112	114	115	119	125	125	0	0	126	134	149	153
15	80B	150	153	0	0	0	0	115	115	121	123	140	148	126	138	0	0
16	88B	0	0	0	0	0	0	115	119	125	125	152	152	122	122	153	157
17	89B	153	159	156	158	112	114	115	119	125	125	148	148	122	122	153	157
18	90B	0	0	0	0	112	114	115	119	125	125	0	0	126	134	149	153
19	96B	129	132	0	0	112	114	115	119	125	125	0	0	146	138	149	153
20	98B	153	153	0	0	112	114	115	119	125	125	0	0	126	134	149	153
21	101B	153	153	156	162	112	114	115	119	125	125	140	148	126	134	149	153
22	106B	150	150	0	0	130	128	119	127	0	0	156	152	146	146	0	0
23	107B	153	153	156	162	112	114	115	119	0	0	0	0	126	134	149	153
24	U16B	153	159	156	156	0	0	115	119	125	125	0	0	126	138	0	0
25	U17B	153	159	0	0	0	0	115	119	125	131	156	156	138	126	0	0
26	121B	129	150	154	156	0	0	0	0	125	139	140	136	126	138	0	0
27	124B	0	0	0	0	132	128	119	115	0	0	140	152	138	146	149	153
28	125B	129	132	154	156	114	112	115	119	125	131	0	0	126	134	161	153
29	126B	150	153	0	0	128	130	0	0	123	129	172	176	0	0	153	161
30	161B	153	159	154	156	0	0	115	119	131	141	0	0	122	122	153	161
31	162B	129	150	154	156	130	132	119	125	0	0	140	156	0	0	153	161
32	163B	150	153	154	156	130	128	0	0	0	0	0	0	126	138	153	161
33	164B	129	150	154	156	130	132	0	0	0	0	140	148	126	138	153	161
34	166B	129	150	154	156	130	128	0	0	0	0	172	172	0	0	153	161
35	167B	129	150	154	156	130	132	0	0	0	0	172	172	126	138	153	161
36	169B	129	132	0	0	0	0	119	125	0	0	156	156	138	146	153	161
37	171B	129	150	0	0	128	130	0	0	125	129	0	0	146	138	153	161

3. Analysis results of the samples.

- Probability of Amur Tiger individual identification

This is the P(ID) result, we can see from the table that the probability of identifying tiger individuals with 10 loci is also high, reaching 7.714e-08, and for sibs, the value is 1.555e-03.

locus	biased/loci	unbias./loc.	sibs/locus	Prod(biased)	Prod(unbias)	Prod(sibs)
E6	3.408e-01	3.238e-01	6.057e-01	3.408e-01	3.238e-01	6.057e-01
E21	2.781e-01	2.614e-01	5.588e-01	9.476e-02	8.464e-02	3.384e-01
D10	4.722e-01	4.527e-01	7.050e-01	4.474e-02	3.831e-02	2.386e-01
FCA304	1.717e-01	1.605e-01	4.600e-01	7.681e-03	6.148e-03	1.097e-01
FCA043	1.708e-01	1.607e-01	4.560e-01	1.312e-03	9.883e-04	5.004e-02
FCA391	9.267e-01	9.230e-01	9.628e-01	1.216e-03	9.122e-04	4.818e-02
FCA152	1.593e-01	1.474e-01	4.525e-01	1.937e-04	1.344e-04	2.180e-02
FCA441	2.821e-02	2.062e-02	3.218e-01	5.465e-06	2.772e-06	7.016e-03
F53	2.895e-01	2.805e-01	5.439e-01	1.582e-06	7.777e-07	3.815e-03
F85	1.098e-01	9.920e-02	4.075e-01	1.737e-07	7.714e-08	1.555e-03

3. Analysis results of the samples.

- The amplificaton result of the 10 locis of amur tiger is acceptable for Amur tigers.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
1		E6		E21		D10		FCA304		FCA043		FCA391		FCA152		FCA441		F53		F85	
2	1H	150	153	156	156	148	148	130	132	125	129	210	210	131	143	156	160	138	146	173	173
3	2H	150	153	156	156	148	148	130	132	125	129	210	210	131	143	156	160	138	146	173	173
4	4H	150	153	156	156	148	148	130	132	119	129	210	210	0	0	148	156	146	146	173	173
5	5H	150	153	156	158	148	148	130	130	0	0	210	210	139	139	156	156	0	0	0	0
6	7H	150	150	156	156	148	150	124	132	125	129	210	210	131	143	156	160	138	138	153	173
7	8H	150	150	156	156	148	150	124	132	125	129	210	210	131	143	156	160	134	138	0	0
8	9H	150	153	156	156	148	150	124	132	125	129	210	210	131	143	156	160	0	0	153	173
9	10H	150	150	160	160	148	148	0	0	125	125	210	210	135	143	148	148	138	146	0	0
10	11H	150	153	156	156	148	150	124	132	125	129	210	210	131	143	156	160	138	138	0	0
11	13H	150	150	156	156	148	148	130	132	0	0	0	0	131	143	156	160	138	146	0	0
12	14H	150	153	0	0	148	148	0	0	119	129	0	0	125	130	0	0	138	146	153	173
13	15H	150	153	156	156	148	148	130	132	119	129	210	210	143	143	0	0	146	146	0	0
14	16H	150	150	156	156	148	148	130	132	119	129	210	210	143	143	152	156	138	142	0	0
15	31H	150	153	156	158	148	148	130	132	119	129	210	210	131	143	0	0	146	150	0	0
16	32H	150	150	156	158	148	148	130	132	119	129	0	0	143	143	0	0	146	146	0	0
17	46H	0	0	158	158	148	148	130	130	119	129	0	0	0	0	0	0	138	146	173	173
18	50H	150	153	156	156	148	148	0	0	125	129	0	0	0	0	0	0	138	146	173	173
19	61H	0	0	0	0	148	148	124	132	118	129	0	0	131	131	0	0	138	146	165	165
20	F4H	150	150	156	158	148	150	124	132	125	129	0	0	131	143	0	0	138	138	153	173
21	F5H	150	150	156	158	148	150	124	132	125	129	0	0	131	143	0	0	138	138	153	173
22	51H	150	153	0	0	150	150	112	130	0	0	0	0	139	139	176	216	138	166	165	165
23	52H	150	150	0	0	148	150	124	130	0	0	0	0	125	125	176	216	138	146	173	173
24	53H	150	153	150	160	0	0	132	120	125	131	0	0	125	143	164	164	138	146	149	153
25	54-1H	150	150	156	158	148	148	130	132	125	129	210	210	131	143	180	180	138	146	149	153
26	54-2H	150	150	156	158	148	148	130	132	125	129	210	210	131	143	176	176	138	146	149	153
27	55H	150	150	156	158	148	148	130	132	125	129	210	210	131	143	176	176	138	146	149	153
28	56H	150	150	156	158	148	148	130	132	125	129	210	210	131	143	200	204	138	146	148	153
29	57H	150	150	156	158	148	148	130	132	125	129	210	210	131	143	172	172	138	146	153	165
30	58H	150	150	156	158	148	148	130	132	125	129	210	210	131	143	0	0	138	146	149	153
31	59H	150	153	156	158	0	0	130	132	127	129	0	0	125	143	172	196	134	138	149	165
32	510H	150	153	156	158	148	148	130	130	129	129	202	210	131	143	180	196	138	146	173	173
33	511H	129	150	156	158	148	148	120	130	119	129	210	210	125	143	200	204	138	138	149	153
34	512H	150	150	156	158	148	148	130	132	125	129	210	210	131	143	188	208	138	146	149	153
35	513H	150	150	156	158	148	148	130	132	125	129	210	210	131	143	176	176	138	146	165	173
36	U08H	150	153	156	160	148	148	124	132	119	129	0	0	0	0	0	0	138	146	153	173
37	U14H	150	153	0	0	148	148	0	0	119	129	0	0	125	130	0	0	138	146	153	173
38	115H	129	150	154	156	148	152	130	132	119	125	0	0	131	141	148	156	138	146	0	0
39	118H	150	129	154	156	0	0	128	130	119	125	0	0	123	139	0	0	138	146	0	0
40	150H	0	0	154	156	148	152	0	0	119	125	210	210	0	0	148	148	138	146	153	161
41	151H	150	153	156	158	148	148	132	132	119	125	210	210	131	143	0	0	138	146	173	173
42	152H	150	153	156	158	148	148	132	132	119	125	210	210	131	143	148	156	138	146	173	173
43	155H	150	129	156	156	148	148	132	132	119	129	0	0	143	143	0	0	138	146	0	0
44	158H	129	150	156	158	148	148	130	132	119	129	0	0	0	0	0	0	0	0	153	161
45	172H	129	150	156	156	162	152	130	128	119	129	0	0	0	0	148	156	0	0	153	161
46	173H	150	150	156	156	148	148	130	132	119	129	210	210	143	143	156	156	138	138	0	0
47	175H	129	150	154	156	148	148	128	130	0	0	0	0	0	0	0	0	138	142	153	161
48	176H	150	150	156	156	148	148	130	132	119	129	0	0	0	0	0	0	138	138	0	0
49	177H	129	150	156	156	148	148	130	132	0	0	0	0	125	143	0	0	146	146	0	0
50	178H	0	0	154	156	148	152	128	130	121	129	0	0	0	0	172	172	0	0	153	161
51	183H	129	150	156	156	152	162	0	0	119	125	0	0	125	129	148	156	0	0	153	161
52	185H	129	150	154	156	148	148	0	0	0	0	0	0	0	0	140	140	138	146	153	161

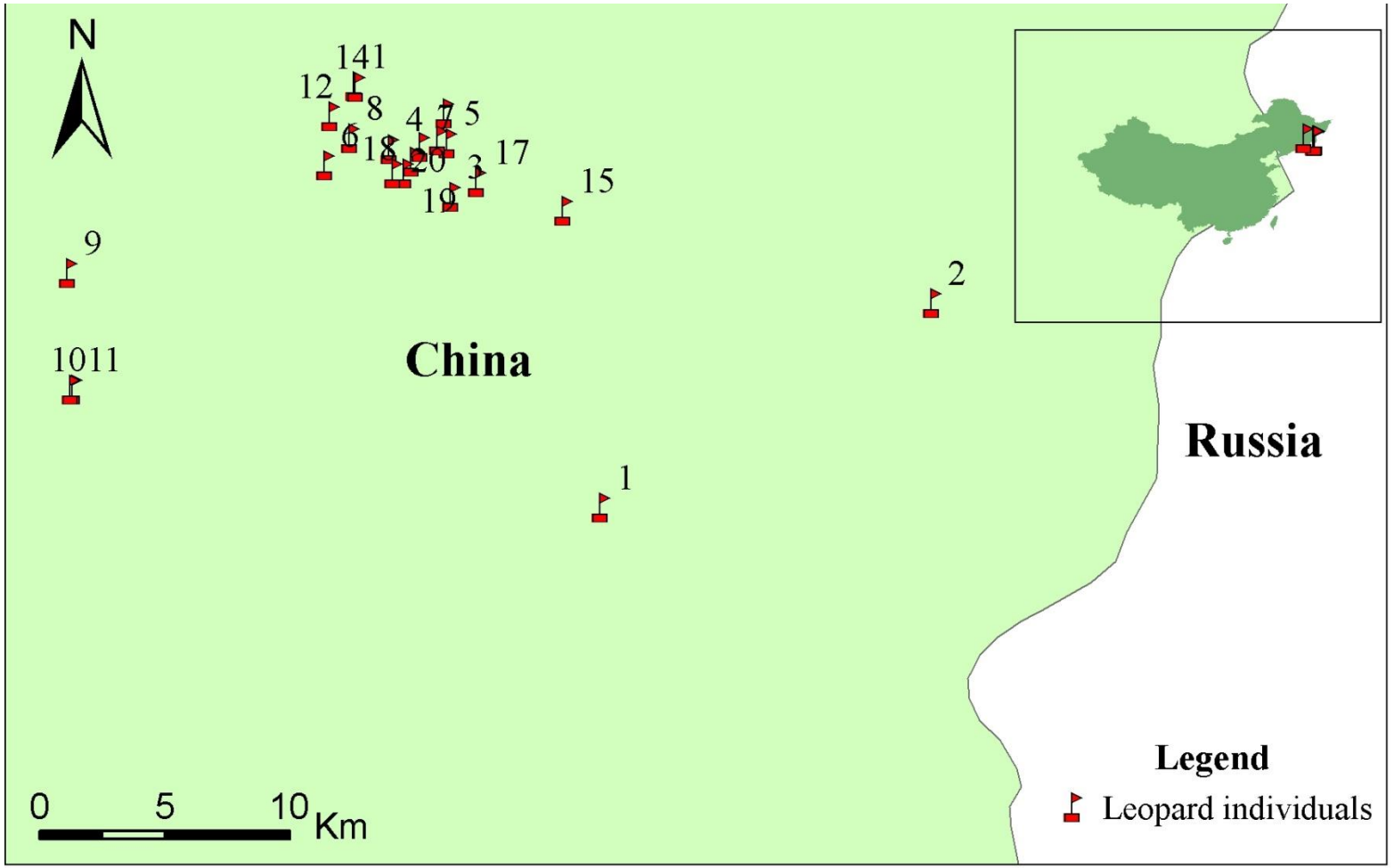
3. Analysis results of the samples.

- Individualization determination principles

Using MStools to distinguish the samples with the same genotype, the main principle is to determine the same individual if all the locis of them are the same or only one loci is different at most.

3. Analysis results of the samples.

- The 36 samples were determined to be 21 Amur leopards and their spatial distribution.



3. Analysis results of the samples.

- Genetic diversity of Amur leopards

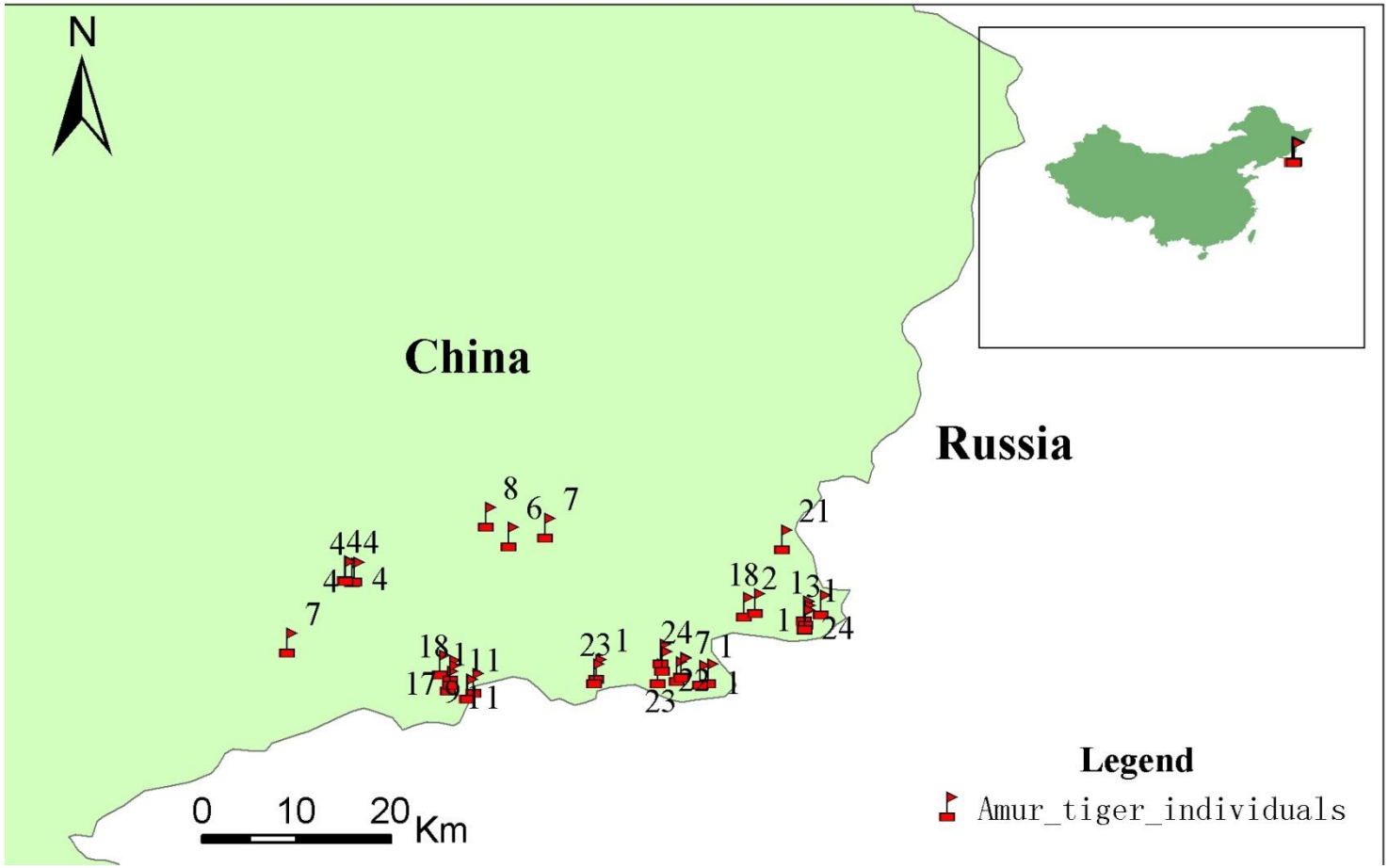
① The Characteristics of the 8 microsatellites in wild leopard population, and their overall HO is higher than the HE.

② Most of the PIC values of microsatellites are above 0.5, which indicates that the microsatellite of leopard has a high polymorphism.

Locus	k	N	HObs	HExp	PIC
E	6	29	0.759	0.732	0.674
E2	5	21	0.952	0.654	0.587
FCA30	5	24	1.000	0.770	0.714
FCA04	4	29	0.931	0.557	0.446
FCA15	7	27	0.296	0.365	0.348
FCA44	8	22	0.500	0.837	0.793
F5	5	29	0.793	0.787	0.738
F8	4	29	0.966	0.653	0.588

3. Analysis results of the samples.

- The 51 samples were determined to be 24 Amur tigers and their spatial distribution.



3. Analysis results of the samples.

① The overall level of HO is greater than HE for Amur tigers.

② The average value of PIC is 0.506, indicating that the loci of tiger has a high polymorphism.

Locus	k	N	HObs	HExp	PIC
E	3	47	0.596	0.464	0.411
E2	5	47	0.596	0.527	0.472
D1	4	48	0.271	0.330	0.308
FCA30	7	45	0.867	0.673	0.606
FCA04	6	43	0.953	0.681	0.609
FCA39	2	26	0.038	0.038	0.037
FCA15	8	40	0.775	0.683	0.621
FCA44	16	33	0.636	0.884	0.859
F5	6	45	0.756	0.563	0.464
F8	5	36	0.694	0.750	0.698

3. Analysis results of the samples.

- Sex determination

Sex primer(56℃annealing)

ZFX-PF（5'-TACCGAGCGATATAGCTCCAG-3'）

ZFX-PR（5'-GTGTTCCTACGTTAAGCTATTG-3'）

DBY7-PF（5'-CTCATGAAGCCCTATTTTTGGTTG-3'）

DBY7-PR（5‘-ACGGCGTCCGTATCTTCCA-3’）

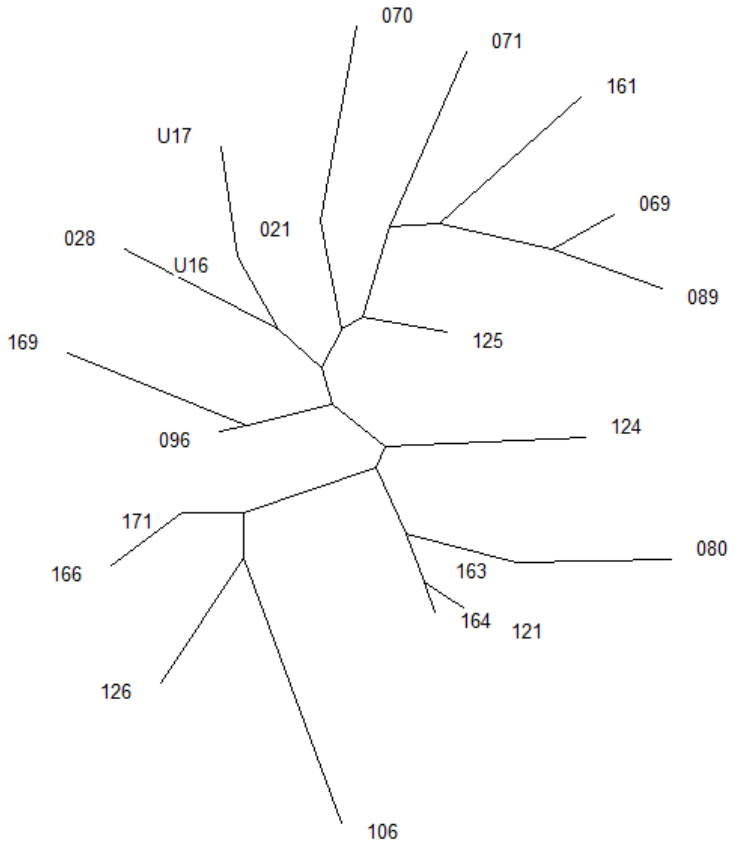
3. Analysis results of the samples.

- This is the results of tigers' and leopard's gender confirmed.

样品编号 Samples	物种 species	性别 Sex	样品编号 Samples	物种 species	性别 Sex
1	tiger	雄性	21	leopard	雄性
7	tiger	雄性	67	leopard	雄性
4	tiger	雌性	162	leopard	雄性
31	tiger	雄性	166	leopard	雌性
175	tiger	雄性	70	leopard	雄性
B1	tiger	雌性	71	leopard	雄性
B3	tiger	雌性	80	leopard	雄性
B10	tiger	雄性	89	leopard	雄性
B13	tiger	雄性	96	leopard	雌性
B11	tiger	雄性	U16	leopard	雌性

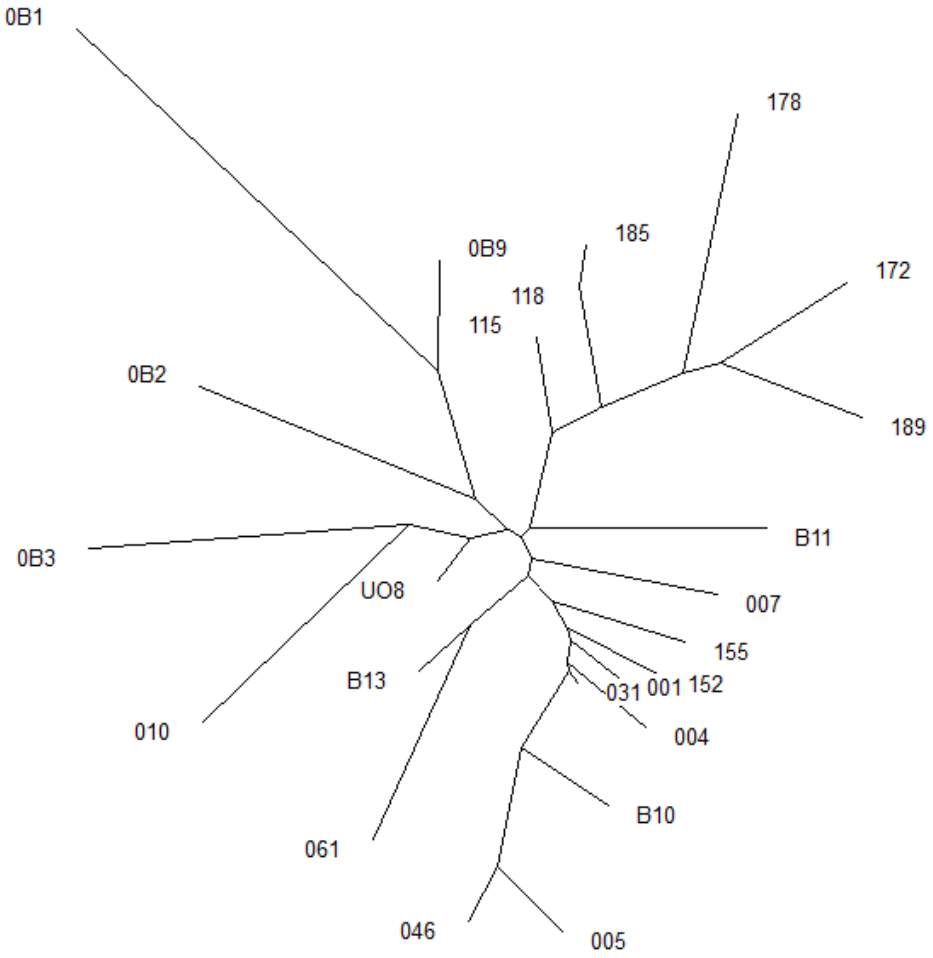
3. Analysis results of the samples.

- Tree of genetic relationship of wild Amur leopard individuals (n=21).



3. Analysis results of the samples.

- Tree of genetic relationship of wild Amur tiger individuals (n=24).



4. Summary

1) Based on this research, we can confirm that at least 21 Amur leopards and 24 Amur tigers are living in China until now;

2) According to the preliminary genetic information, we also know that the population of tiger and leopard have a high gene diversity;

3) Sex can be determined by non-invasive methods;

4) Relationships among individuals can be confirmed across the range;

5) Need to compare the individuals near the Sino-Russia border areas to confirm the corridor positon and continue to collect the samples in field for disease, gene and food habitat detections in future.


T!
THANK YOU FOR YOUR ATTENTION
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