

NEASPEC Project, “Study on Transborderly Movement of Amur Tigers and Leopards using Camera Trapping and Molecular Genetic Analysis”

Applying Molecular Genetic Analysis for the Monitoring of Amur Leopard Population and Their Movement in Transboundary Region

April 15, 2014

Conservation Genome Resource Bank for Korean
Wildlife (CGRB)

Seoul National University

Background

- The current methods used to estimate Amur tiger and leopard populations are snow track counting and camera trapping. Each of these methods has several advantages and drawbacks.
- Individual identification and population size estimation by microsatellite loci analysis using tiger/leopard scat DNA has important advantages that would supplement traditional population monitoring techniques.
 - Statistically robust and enables accurate population estimation by individual identification and sex ratio determination.
 - Provides additional information on genetic diversity of populations, relationships between individuals, population structure and gene flow between populations.

Purpose of the Study

- Assess the transborder movement of Amur leopards between Hunchun, China and southwest part of Primorsky, Russian Far East
- Estimate the population size and home range size
 - using non-invasive genetic sampling and molecular genetic techniques.
 - simultaneously investigate the genetic diversity, levels of inbreeding, and family relationships between individuals.

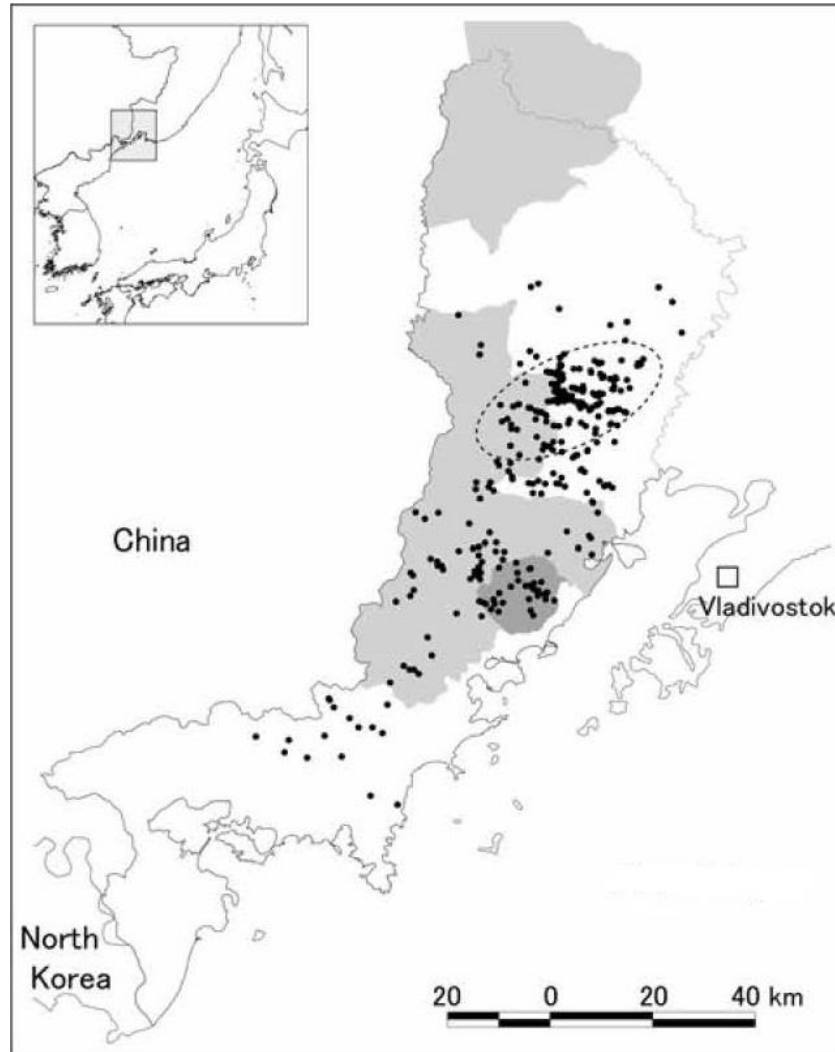
Previous Studies

- There have been continuous research efforts to apply non-invasive genetic sampling and molecular genetic techniques to tiger and leopard population monitoring since the 2000s, and currently the techniques are mature enough to be practical (Perez et al., 2006; Bhagavatula and Singh, 2006; Mondol et al., 2009; Henry et al., 2009).
- Sugimoto et al. (2012) determined genotypes of 10 microsatellite loci using non-invasive samples of Amur tigers like feces, hairs and saliva, which was collected in southwestern part of Primorsky, Russian Far East for four winter periods from 2000 to 2005.

Amur Leopard Population Monitoring by Noninvasive Genetic Analysis by Sugimoto et al. (2013)

- Sugimoto et al. (2013) determined genotypes of 13 microsatellite loci using non-invasive samples of Amur leopards like feces, hairs and saliva, which was collected in Land of the Leopard for 7 winter periods from 2000 to 2008.
- Of total 472 samples collected, 239 were from leopards (50.6%). Of the 239 leopard samples, genotypes were determined for 155 samples (64.9%) and 37 individuals were identified (18 males and 19 females).
- The size of the tiger population estimated by the genetic mark-recapture model was 28 (19-38; 2002-2003 winter) and 26 (2007-2008 winter).
- Half of the individuals were identified from a single sample each.

Map of Sampling Sites



Summary of Sample Information

Table 1 Summary of samples collected and sampling methods in the present study

Winter	No. of sample		Sampling period	No. of personnel	Sampling method	Sampling range
2000–2001	15	(13F, 1H, 1S)	Jan–Mar, May	4	OP, FT	–
2001–2002	102	(91F, 6H, 5S)	Nov–Mar	30	OP, FT	LR
2002–2003	104	(102F, 2H)	Nov–Feb	30	OP, FT	LR
2004–2005	65	(37F, 28H)	Jan–Apr	4	OP, FT, HT	HDA
2005–2006	12	(12F)	Mar	2	SDD	–
2006–2007	86	(80F, 6H)	Oct, Dec–May	10	OP, FT, SDD	LR excluding HDA
2007–2008	88	(87F, 1H)	Dec–Mar	16	OP, FT	LR

F feces, *H* hairs, *S* saliva, *OP* opportunistic, *FT* following the tracks of large cats, *HT* hair traps, *SDD* scat detection dogs, *LR* leopard range except the southernmost area, *HDA* high track density area

Genetic Diversity

Table 2 Genetic variation at 13 microsatellite loci for the 32 individuals identified during the five recent winters (2002–2003 to 2007–2008)

Locus	<i>A</i>	<i>A_E</i>	<i>H_E</i>	<i>H_O</i>	<i>P_{ID-sib}/locus</i>	ADO	FA
FCA026	4	2.36	0.58	0.63	0.52	0.020	0.005
FCA229	3	2.26	0.57	0.56	0.54	0.078	0.025
FCA096	3	2.25	0.56	0.47	0.55	0.147	0.016
FCA211	3	2.17	0.55	0.47	0.56	0.057	0.003
FCA098	2	1.99	0.51	0.56	0.60	0	0.005
FCA043	2	1.93	0.49	0.50	0.60	0.061	0
FCA090	2	1.82	0.46	0.56	0.63	0.033	0
FCA247	2	1.60	0.38	0.31	0.68	0.185	0.010
FCA224	2	1.60	0.38	0.44	0.68	0.146	0.009
FCA105	2	1.48	0.33	0.34	0.72	0.083	0.003
FCA008	3	1.37	0.27	0.31	0.76	0.040	0.002
FCA097	3	1.29	0.23	0.22	0.79	0.081	0.026
FCA123	3	1.29	0.23	0.25	0.79	0.072	0.017
Average	2.62	1.80	0.43	0.43		0.084	0.010

Individual Identification

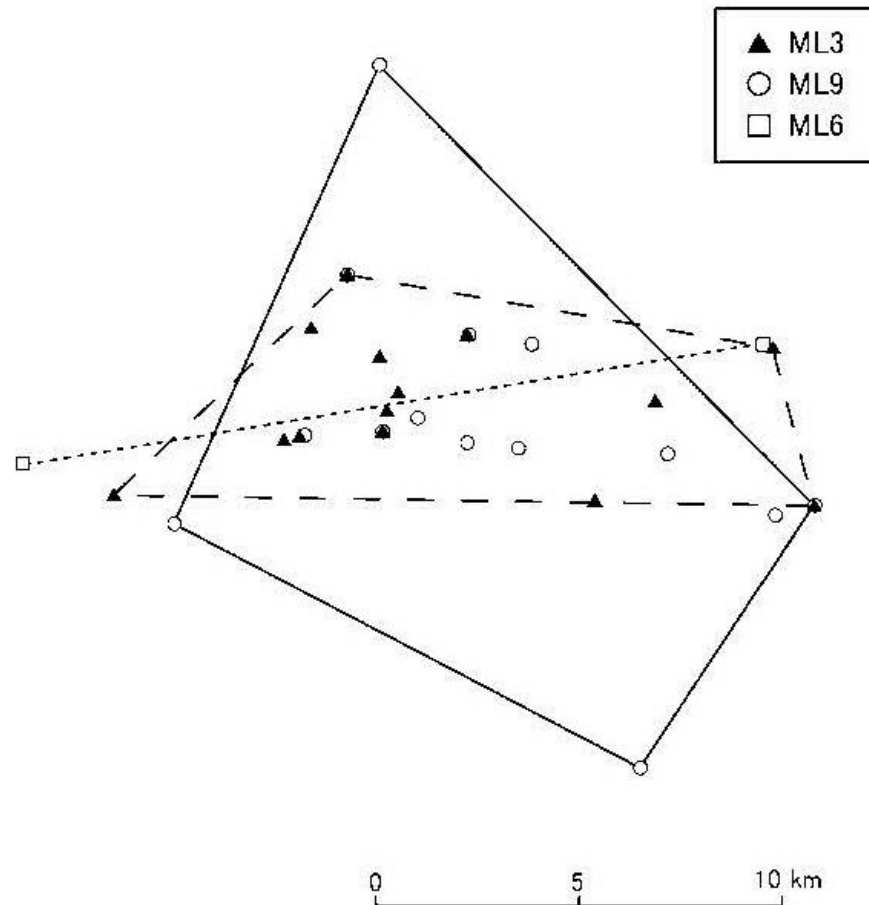
Conserv Genet

Table 3 Results of individual identification for the noninvasive genetic samples collected during the seven winters

Individuals	Total no. of observation	Winters observed						
		2000–2001 (n = 9)	2001–2002 (n = 59)	2002–2003 (n = 58)	2004–2005 (n = 31)	2005–2006 (n = 9)	2006–2007 (n = 24)	2007–2008 (n = 49)
ML1	1	1						
ML2	4		4					
ML3	39		10	8	5	2		14
ML4	4		2	2				
ML5	13		4	9				
ML6	11		2	4	3			2
ML7	10			2			7	1
ML8	6			6				
ML9	19			1		4		14
ML10	2			2				
ML11	1			1				
ML12	5			1			4	
ML13	1			1				
ML14	1			1				
ML15	1				1			
ML16	2						1	1
ML17	1						1	
ML18	1							1
FL1	3	1	2					
FL2	1		1					
FL3	3		1	1	1			
FL4	2		1	1				
FL5	1		1					
FL6	2			2				
FL7	3			2	1			
FL8	2			1				1
FL9	3			2	1			
FL10	1			1				
FL11	3				2			1
FL12	1						1	
FL13	1						1	
FL14	1						1	
FL15	1							1
FL16	1							1
FL17	1							1
FL18	1							1
FL19	1							1
Total	154	2	28	48	14	6	16	40
(n feces/n hairs)	(151/3)	(2/0)	(27/1)	(48/0)	(13/1)	(6/0)	(16/0)	(40/0)

The individuals were labeled as ML# (male leopard no.) and FL# (female leopard no.) in the order of their sampling date. Numbers in parentheses below the winters observed indicate the number of leopard samples revealed from species identification

Home Range Overlap of Two Male Individuals, ML3 and ML9



Limits of Study by Sugimoto et al.

- Sample collection distributed over 8 year period
- No samples from Chinese side and southern part of the Land of the Leopard
- Opportunistic sample collection, by following large cat tracks, or by using scat detection dogs

Conservation Genome Resource Bank for Korean Wildlife (CGRB) Experience In Conservation Genetics

- Individual identification, sex determination, estimation of genetic diversity, phylogeography and population studies through genetic analysis of wildlife in East Asia
 - Determining the subspecific status of Korean tigers using ancient DNA (MtDNA; ; Lee et al., 2012) and tiger genomics study (Cho et al., 2013)
 - Individual identification of river otter using feces (Microsatellite; Park et al., 2011)
 - Sex determination, individual identification, family relationship and genetic diversity study of Asiatic black bears reintroduced in Jirisan Mountain (mtDNA, microsatellites; Kim et al., 2011)
 - Phylogeography of raccoon dogs (MtDNA; Kim et al., 2013)
 - Microsatellite marker developments (Kim et al., 2012; An et al., 2010a, 2010b, 2010c)
 - Forensic science and determining the origin of individuals (Lee et al., 2011a, 2011b; Baker et al., 2010)

Are the Amur tiger in Russian Far East and the Korean tiger the identical subspecies or separate entity?

- *Panthera tigris altaica* vs *Panthera tigris coreensis*
- One way to resolve the problem is to compare the DNA of Korean tigers with DNA of Amur tigers.



Photo by]

[Mr Ford G. Barclay.

CHIN-DŌ, S. KOREA.

PLATE LXXXIV.



Photo by]

[Mr Ford G. Barclay.

PI-NE-SAN, NEAR MOKPO.

PLATE LXXXV.

Yamamoto: A rich Japanese who came to Korea to hunt tigers and leopards in 1917 (during Japanese Colonial Period, 1910-1945)



Hunters and a dead leopard



Hunters and their prey



A Skull Specimen of Korean Tiger

in the National Museum of Nature and Science, Japan



M871, Male, *Panthera tigris altaica*, Meiji 22 (1889) National Museum of Nature and Science , Original specimen collector = Maeda Kenkiochi



Korean tiger skeleton specimens in the Smithsonian Museum, USA



Korean tiger specimens in Doshisha Highschool Museum, Kyoto



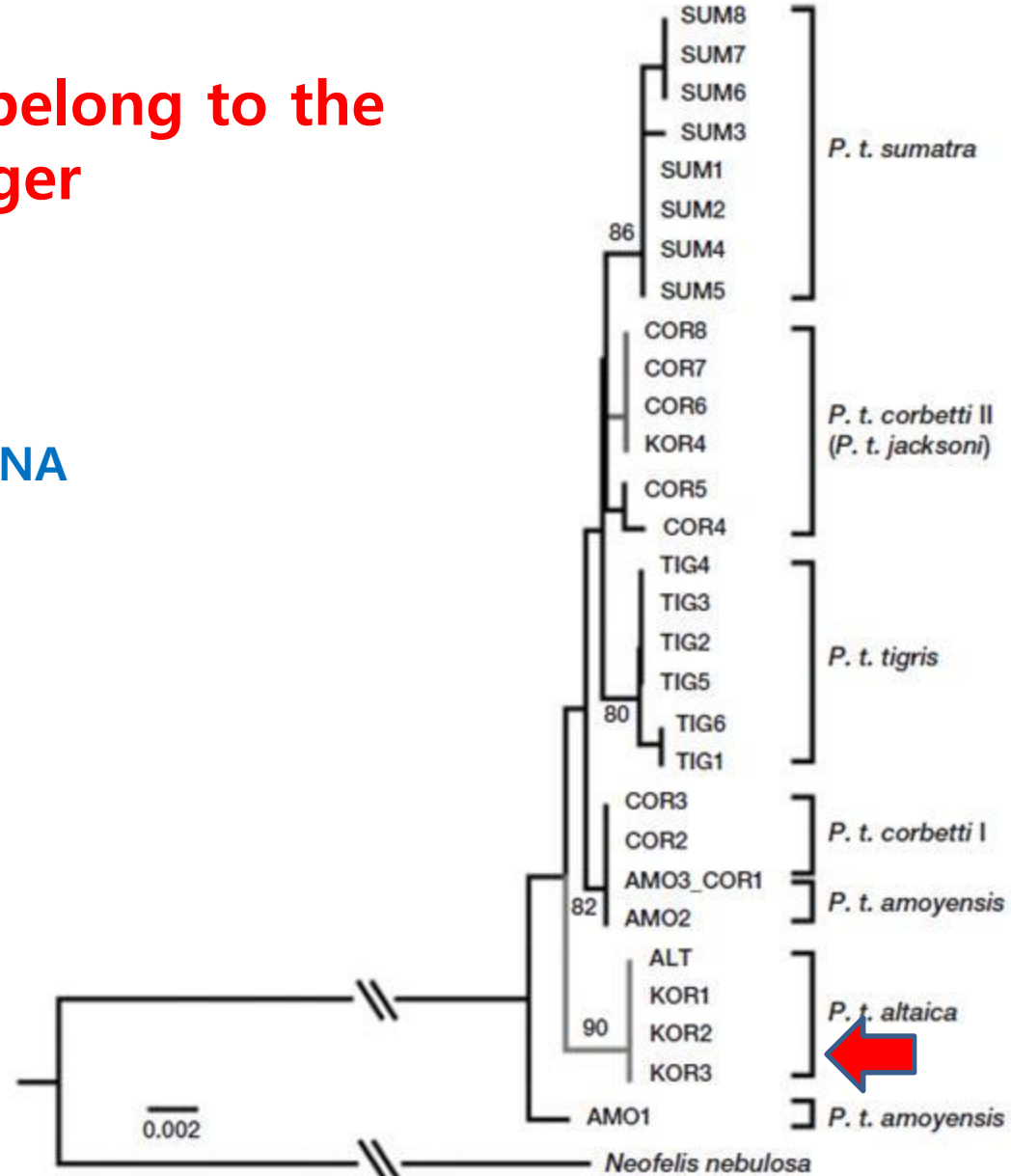
Genetically, Korean tiger belong to the same group with Amur tiger

ML tree (26 seq / 1,052 bp)

Model selection: HKY+G

-ln L=2013.19593

Concatenated 5 fragments of MtDNA
(COI, Cyt *b*, ND2, ND5, ND6)



Subspecific Status of the Korean Tiger Inferred by Ancient DNA Analysis

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ABSTRACT

The tiger population that once inhabited the Korean peninsula was initially considered a unique subspecies (*Panthera tigris corensis*), distinct from the Amur tiger of the Russian Far East (*P. t. altaica*). However, in the following decades, the population of *P. t. corensis* was classified as *P. t. altaica* and hence forth the two populations have been considered the same subspecies. From an ecological point of view, the classification of the Korean tiger population as *P. t. altaica* is a plausible conclusion. Historically, there were no major dispersal barriers between the Korean peninsula and the habitat of Amur tigers in Far Eastern Russia and northeastern China that might prevent gene flow, especially for a large carnivore with long-distance dispersal abilities. However, there has yet to be a genetic study to confirm the subspecific status of the Korean tiger. Bone samples from four tigers originally caught in the Korean peninsula were collected from two museums in Japan and the United States. Eight mitochondrial gene fragments were sequenced and compared to previously published tiger subspecies' mtDNA sequences to assess the phylogenetic relationship of the Korean tiger. Three individuals shared an identical haplotype with the Amur tigers. One specimen grouped with Malayan tigers, perhaps due to misidentification or mislabeling of the sample. Our results support the conclusion that the Korean tiger should be classified as *P. t. altaica*, which has important implications for the conservation and reintroduction of Korean tigers.

Keywords: *Panthera tigris corensis*, phylogenetic relationship, mitochondrial DNA, Korean tiger, *Panthera tigris altaica*, Amur tiger

INTRODUCTION

The tiger, *Panthera tigris*, is one of the most endangered species in the world. In the 1900s, an estimated 100,000 tigers inhabited much of the Asian continent. However, today only 3,000 survive in limited areas (Driscoll et al., 2009; Tilson and Nyhus, 2010). Tigers have been traditionally classified into eight subspecies based upon geographic distribution and morphological differences. Recently a comprehensive molecular analysis of a tiger subspecies using mtDNA and microsatellite genotypes affirmed the distinctiveness of these sub-

species and identified a new subspecies, the Malayan tiger, *P. t. jacksoni* (Luo et al., 2004, 2008). Of these recognized subspecies, three are extinct, *P. t. sondica*, *P. t. balica*, and *P. t. virgata* (Driscoll et al., 2009) and one, *P. t. amoyensis*, only survives in zoos (Tilson et al., 2004; Morell, 2007; Luo et al., 2008).

From the Joseon Dynasty to the Japanese colonial period, tigers were seen as a threat to the Korean people and there was a lot of pressure to eliminate tigers from the wild (Endo, 2009; Kim and Lee, 2011). As a consequence, tigers that had once roamed the forests of the Korean peninsula became



Panthera tigris altaica

Cover Story

A photo of an Amur tiger (*Panthera tigris altaica*) in its wild habitat of Russian Far East by Sooyong Park (see page 51)

The Korean Society of Systematic Zoology

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Counting the number of river otters in Daegu City with genetic analysis of spraints

- Properties Eurasian otter spraints

- Easy
- Distinct

- Show invasive
- Difficult
- Contaminated
- Several false

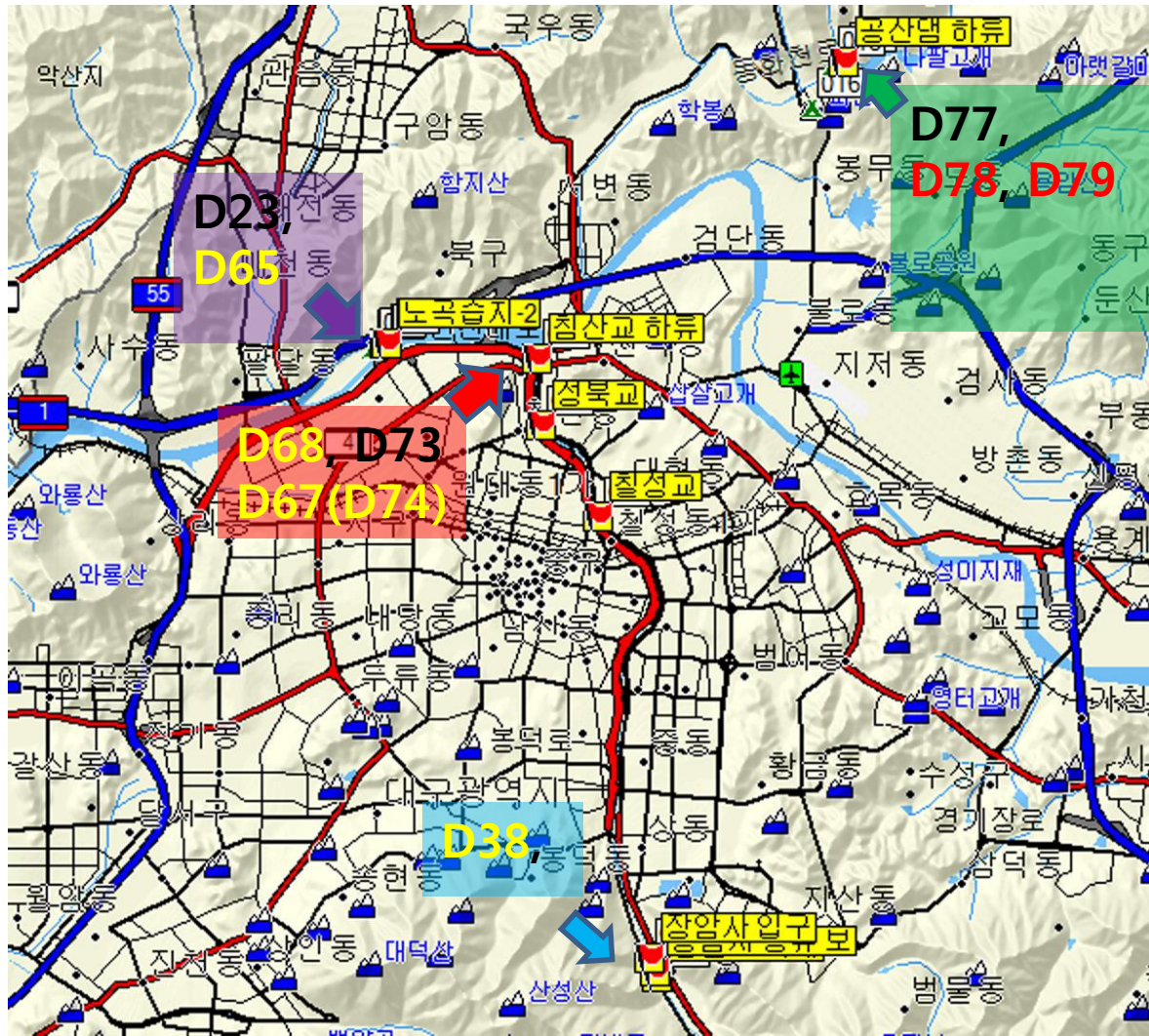


on-

p out,

River Otters in Daegu

- Location of individuals.



Individual Identification of Korean Goral (*Naemorhedus caudatus*)



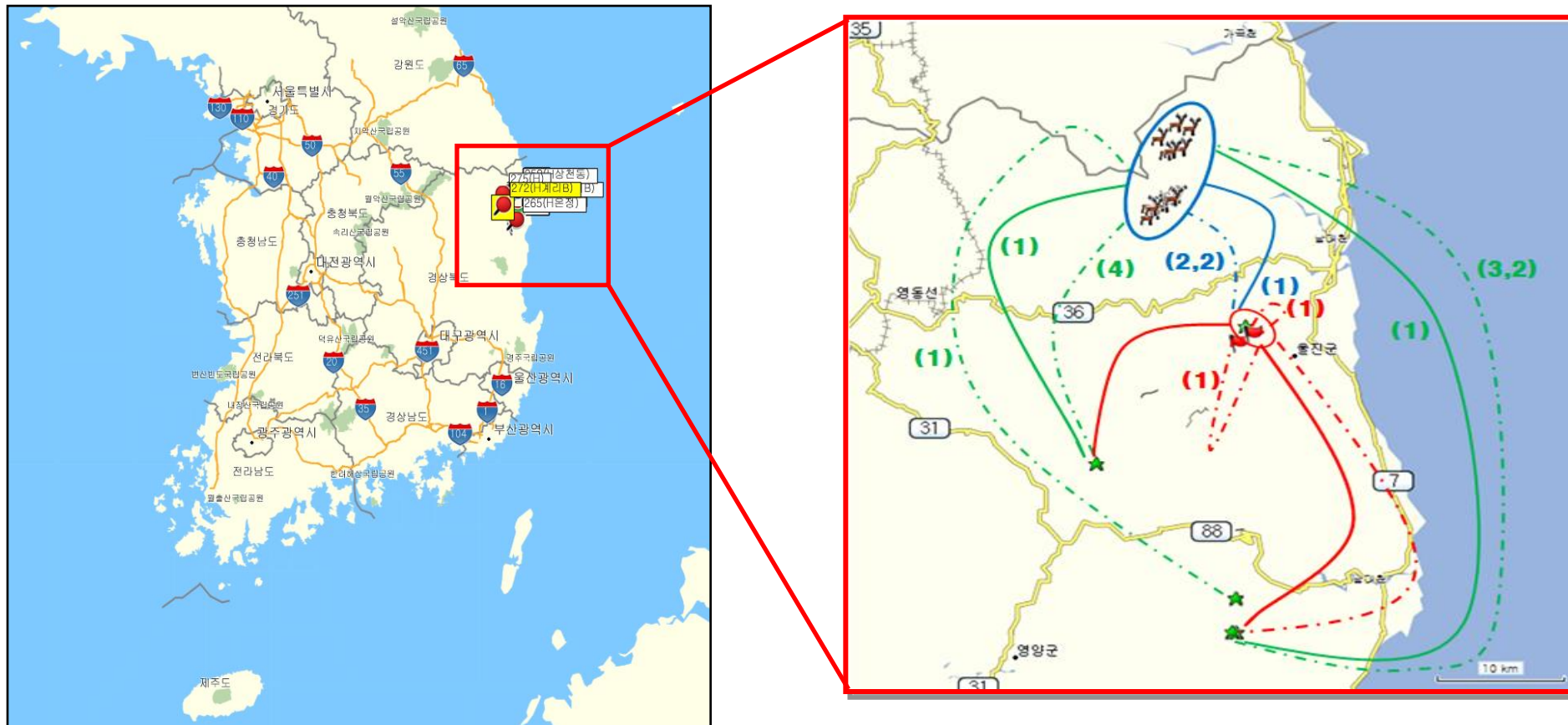
- Microsatellite analysis using non-invasive samples from 2008 to 2010



Korean Goral (*Naemorhedus caudatus*)



- Kinship relationships among identified individuals



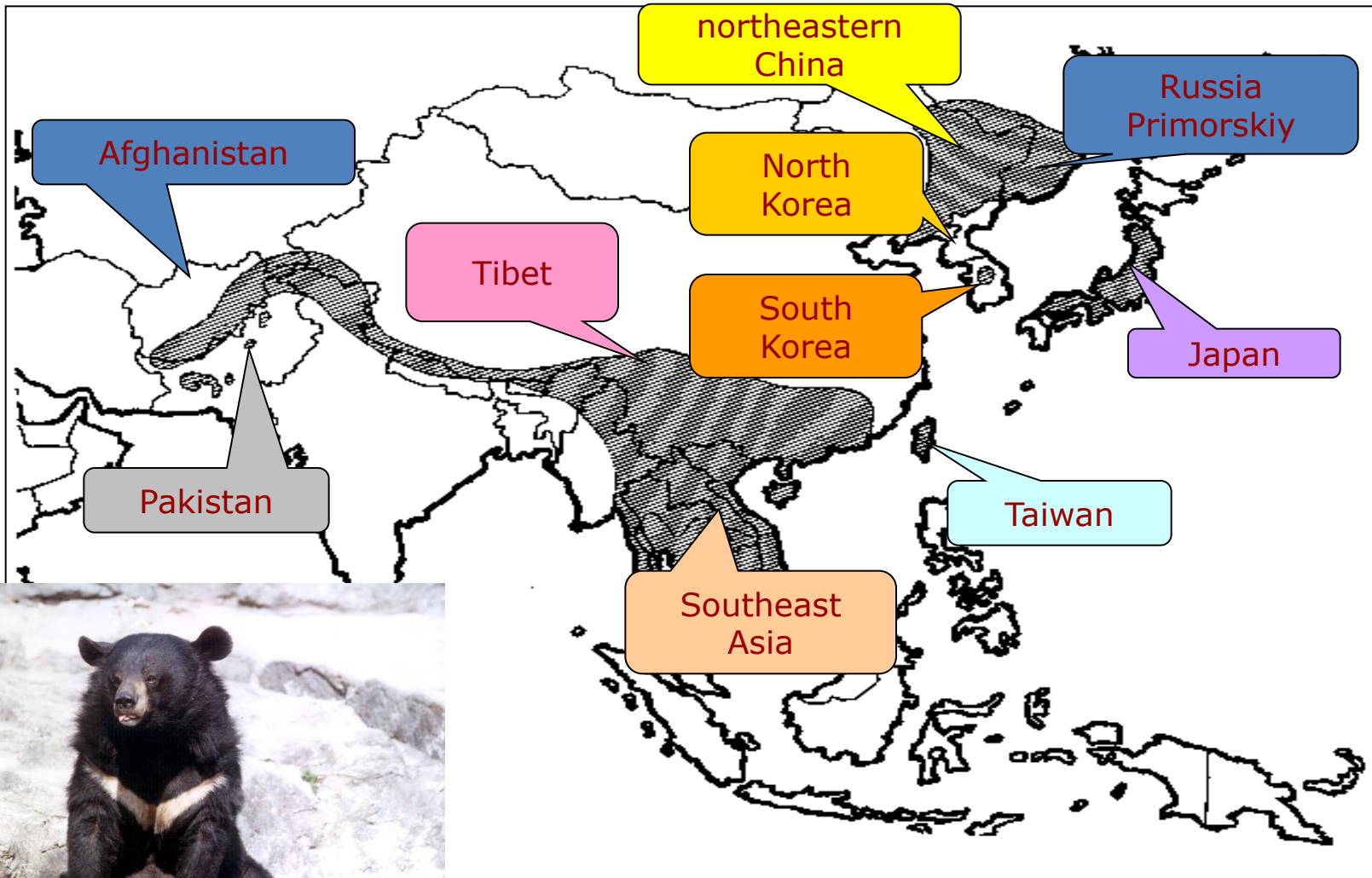
Genetic diversity and phylogeography of Asiatic Black Bear



Asiatic Black Bears reintroduced in Jirisan

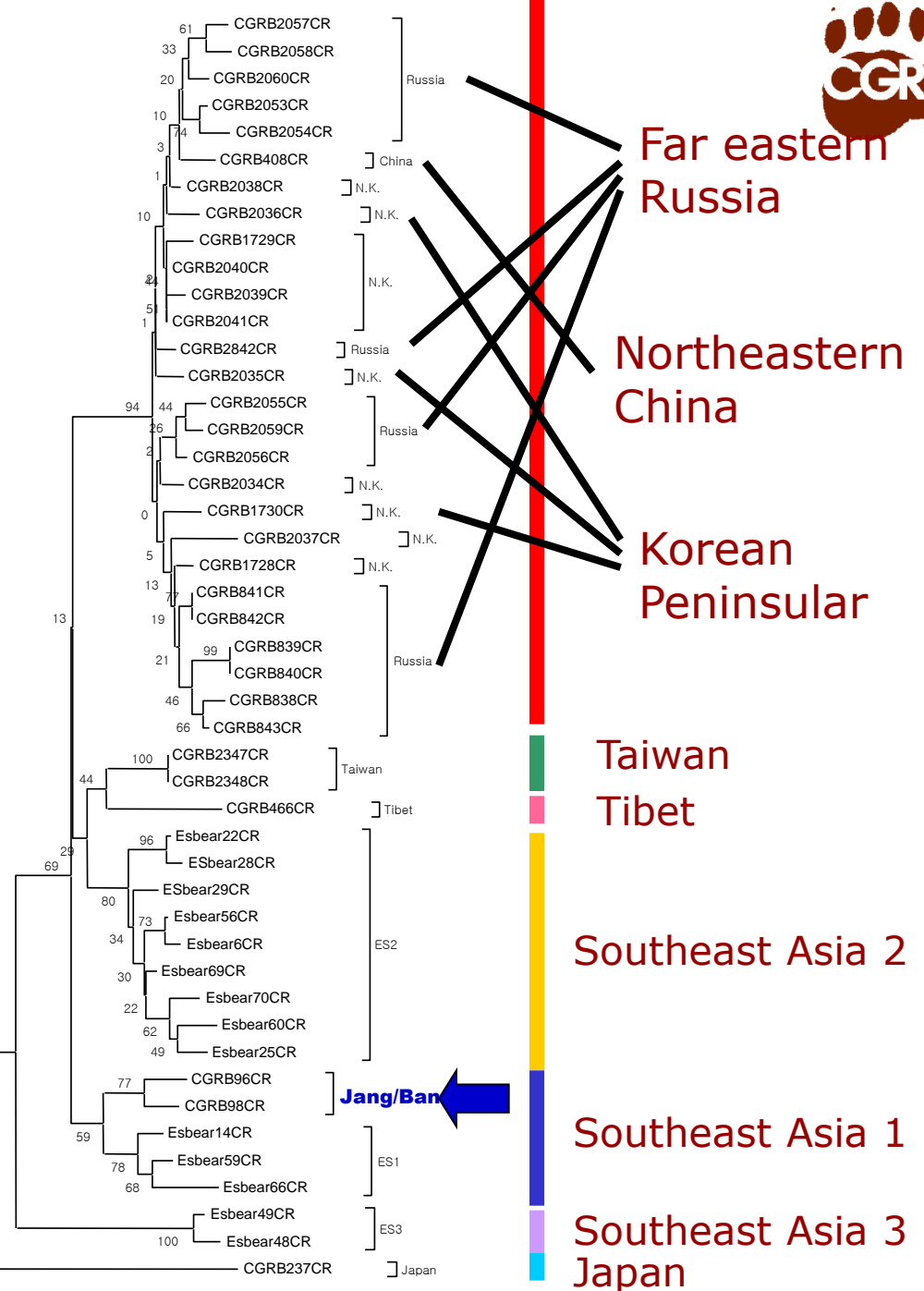


Distribution of Asiatic Black Bears



Conservation Units of Asiatic Black Bear

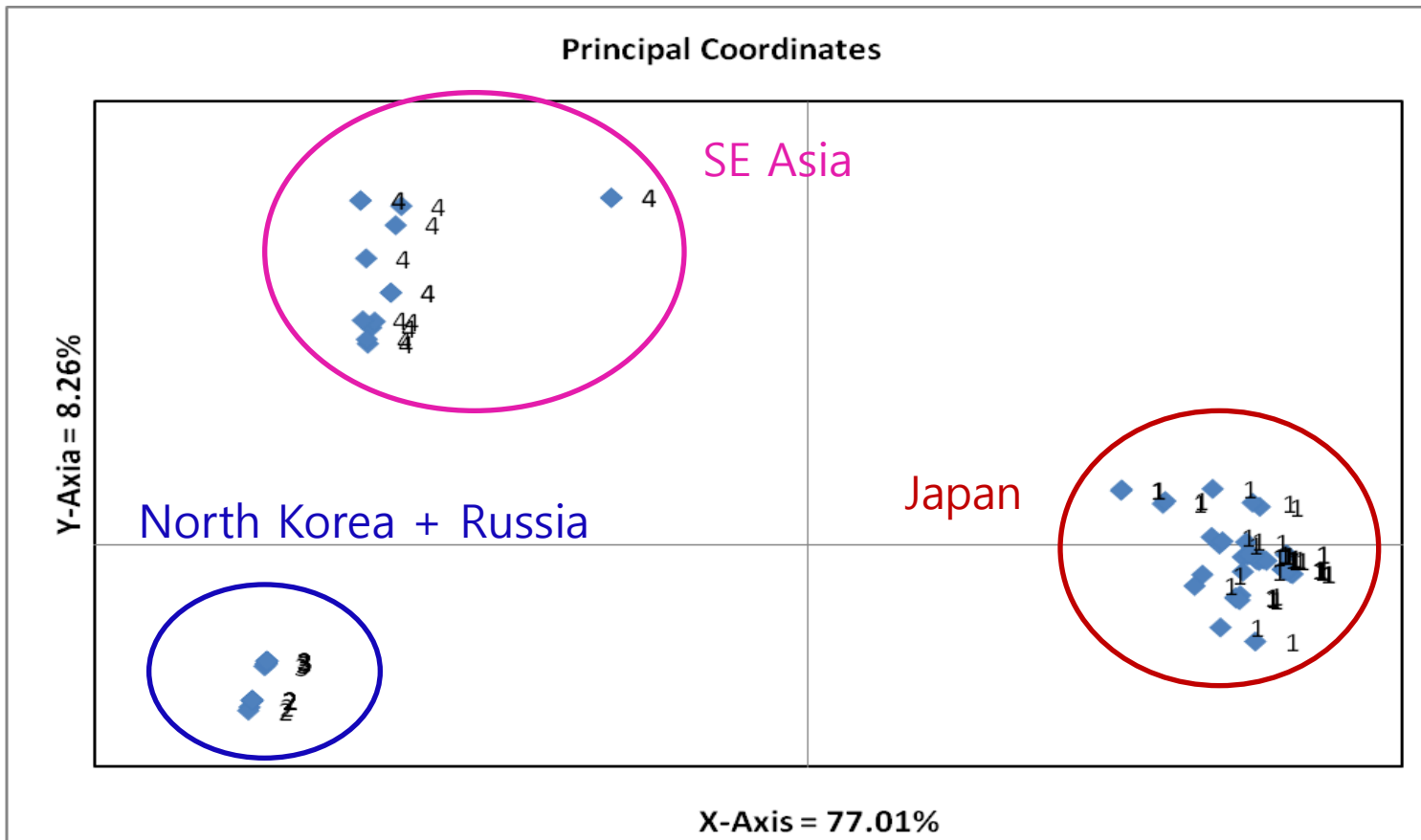
Control Region
 (Neighbor Joining, 1000 Bootstraps)



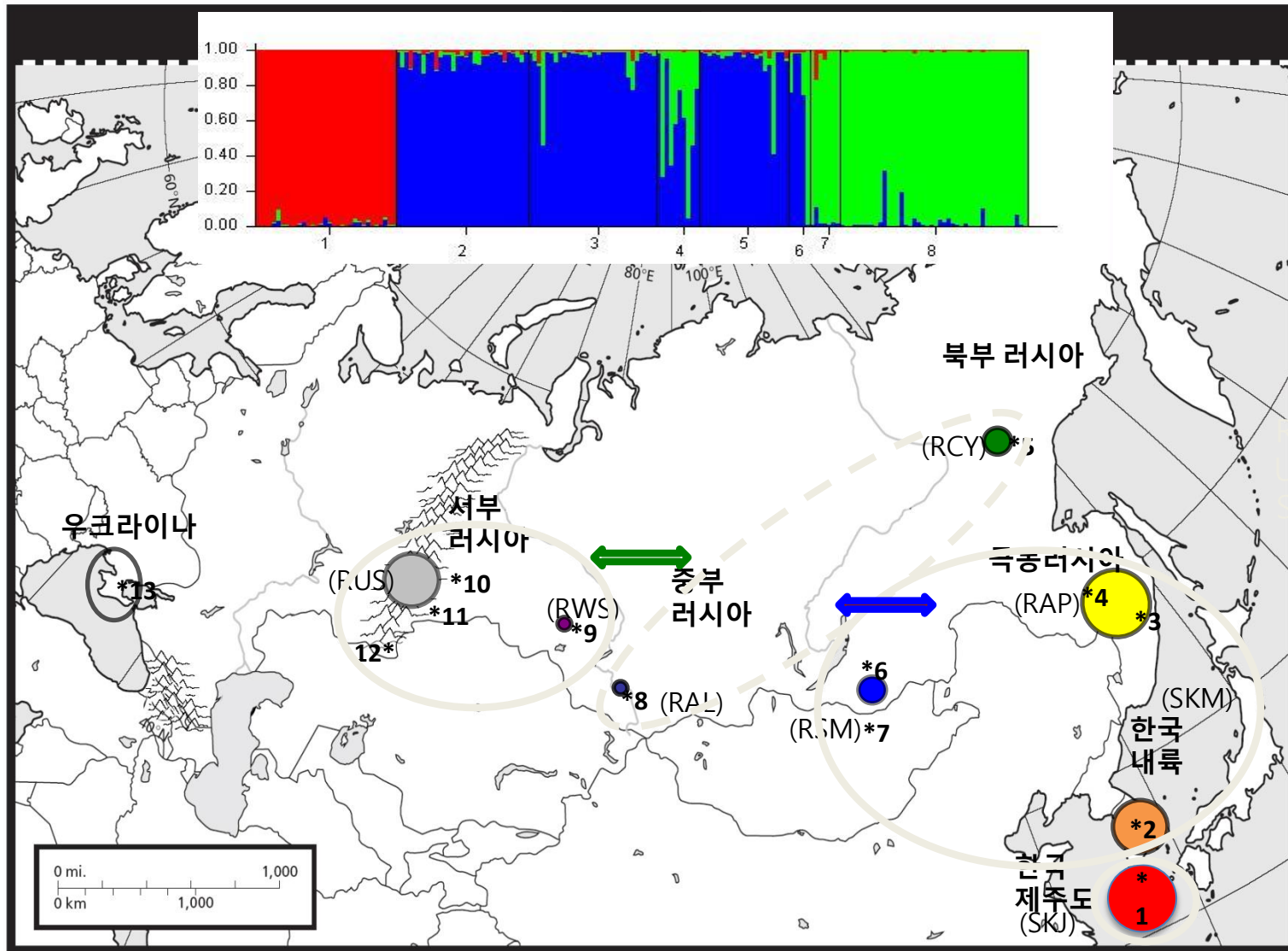
PCA analysis (mtDNA D-loop 615 bp)



사진 출처: 국립공원



Population Structure of Eurasian Roe Deer by Microsatellite Markers



Wild Boar (*Sus scrofa*)



Kingdom: Animalia

Phylum: Chordata

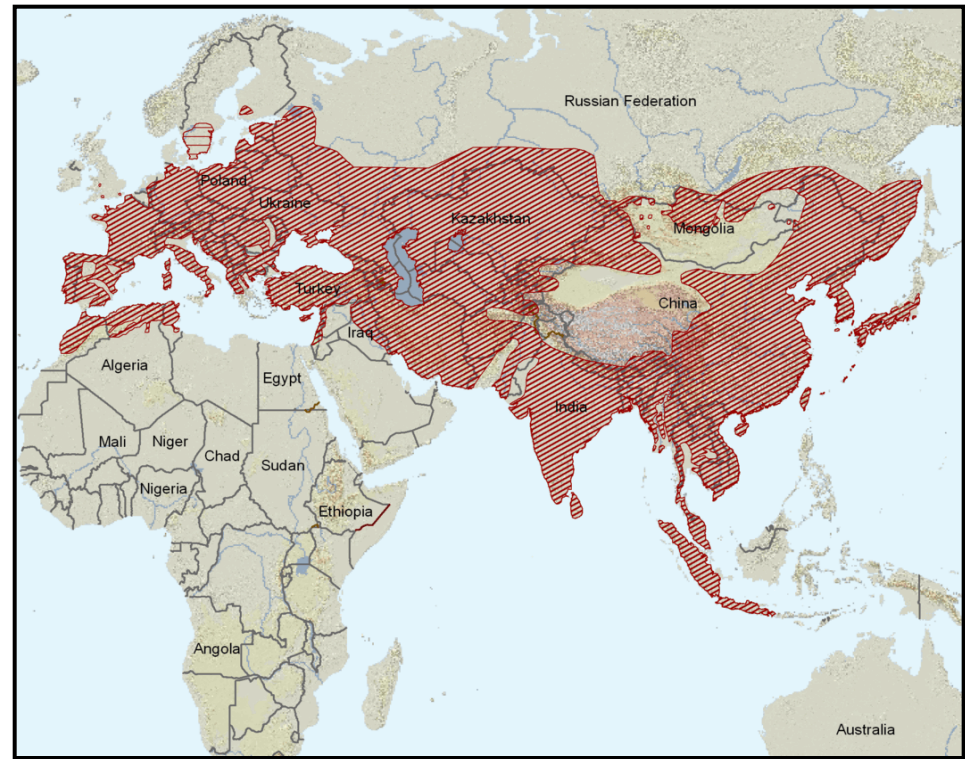
Class: Mammalia

Order: Artiodactyla

Family: Suidae

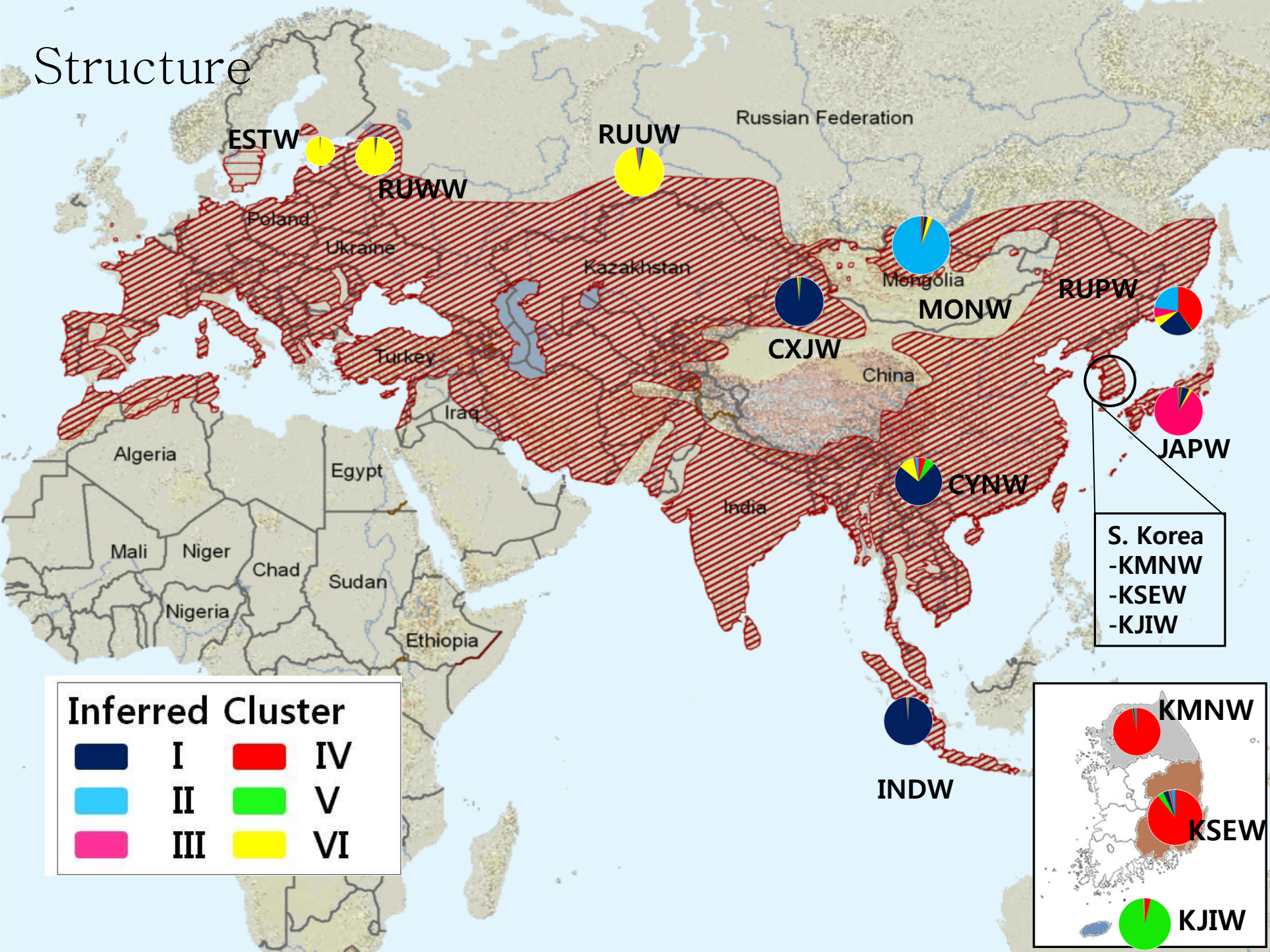
Genus: *Sus*

Species: *S. scrofa*



(IUCN 2008, www.iucnredlist.org)

Structure

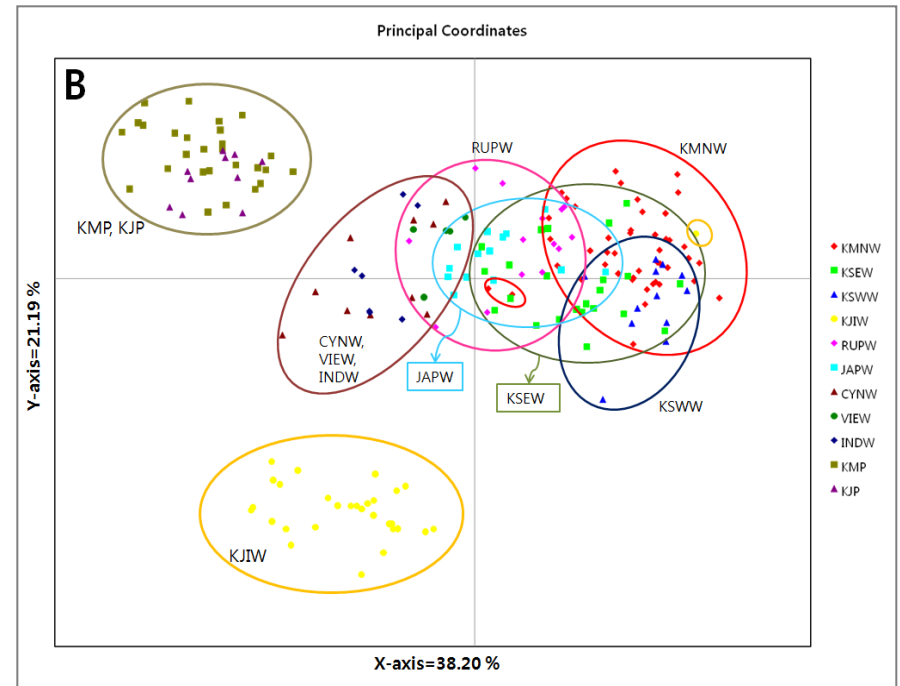
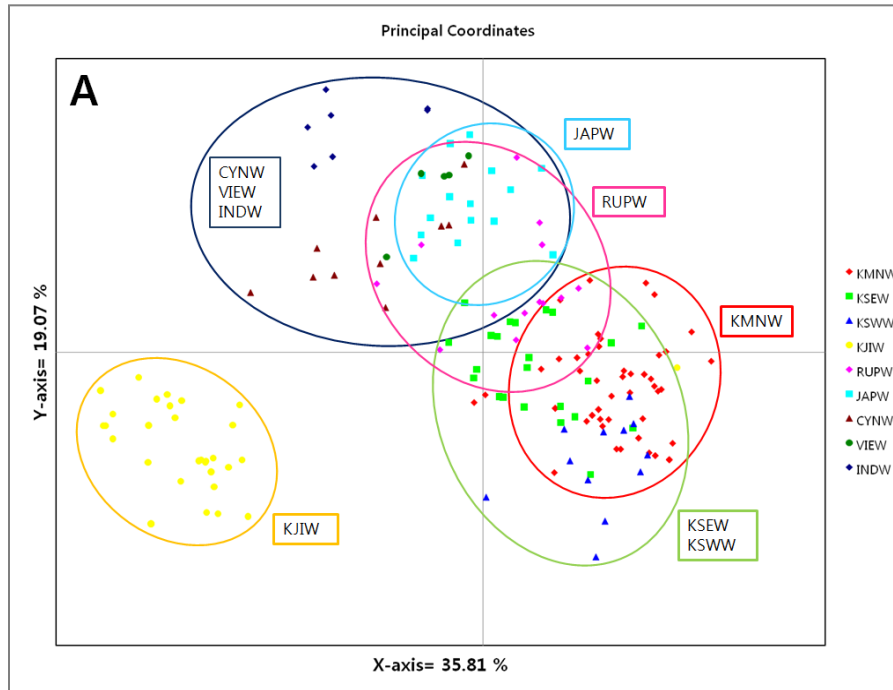


Wild Boar (*Sus scrofa*)



- Microsatellite analysis

- Principal Coordinates Analysis (PCA)



Research Plan

Monitoring of Amur Leopard Population and Their Movement in Transboundary Region Using Molecular Genetic Analysis

- Study Species and Populations
 - Amur leopard (*Panthera pardus orientalis*)
 - in and around the “Land of Leopards” in southwest of Russian Far East and Chinese border area



«LAND OF THE LEOPARD»
 NATIONAL PARK

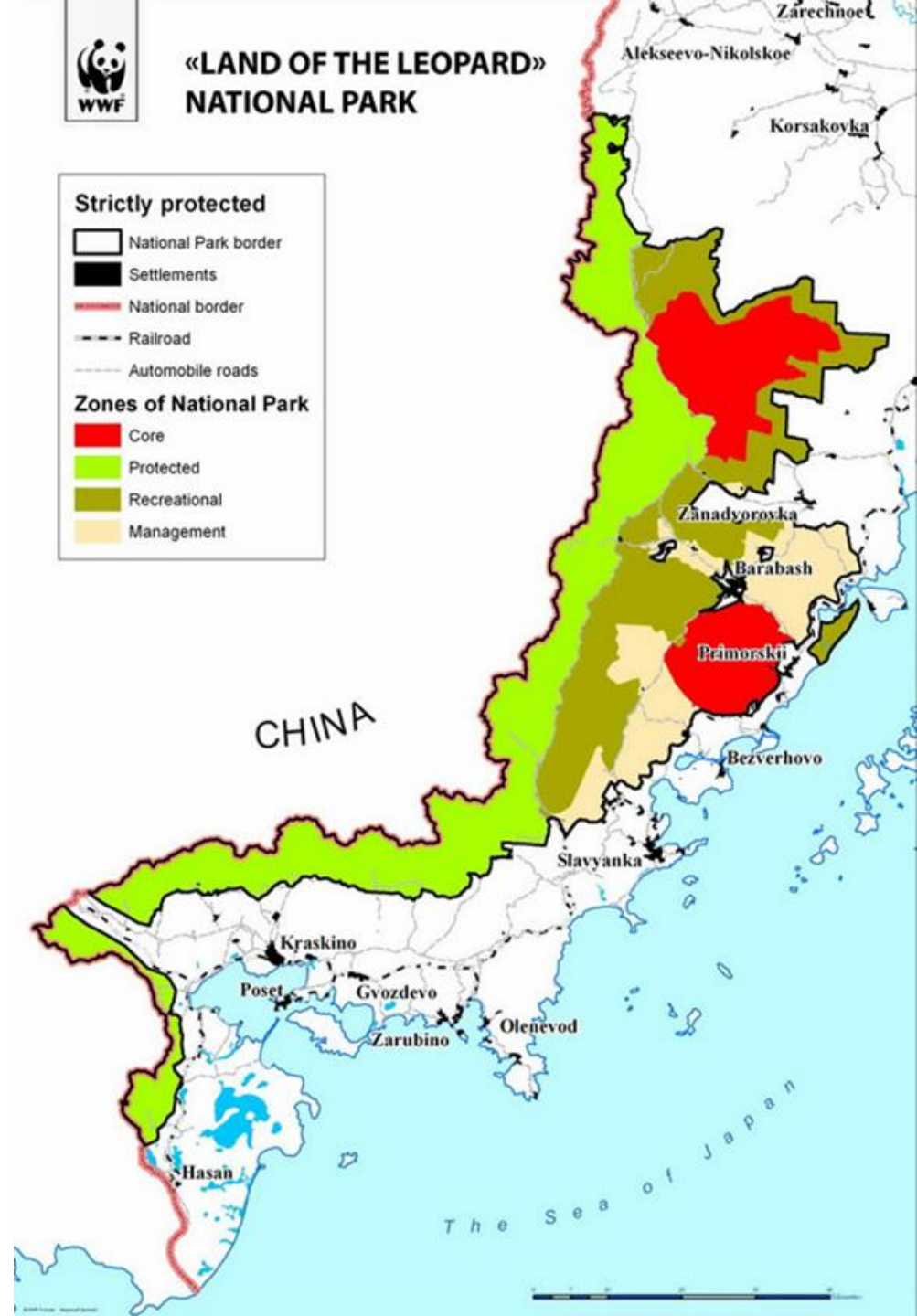
Map of the “Land of the Leopard National Park”

Strictly protected

- National Park border
- Settlements
- National border
- Railroad
- Automobile roads

Zones of National Park

- Core
- Protected
- Recreational
- Management



Fecal sample Collection and DNA extraction

- Sample collection will be done mainly by researchers, rangers and inspectors.
- Two researchers of CGRB will stay in the study area for 2-3 months and assist collecting tiger/leopard feces samples and extract DNA from the samples.
- Collecting non-invasive specimens in the winter period would help to preserve DNA at the naturally low ambient temperature.
- We will not attempt to collect hairs using hair traps because the efficiency of collection was reported to be very low (Sugimoto et al., 2012).
- Based on the data by Sugimoto et al. (2013), the target number of samples to be collected will be set as >400.
- If 400 samples are collected in the field, it is expected that at least 200 (50%) would be from leopards, 100 (25%) from tigers, to be determined by species identification. About 80% of these species-identified samples are expected to be individually identified by microsatellite loci typing (leopards, 160; tigers, 80). Thus, total number of collected sample 400 is required to secure minimum observation number (>3 times of assumed population size) to accurately estimate leopard and tiger population sizes by mark and recapture model (Capwire model; Miller et al., 2005).
- The samples need to be collected as evenly as possible covering whole range including Chinese side.
- The collected specimens will be stored at -20°C until DNA extraction. DNA extraction will be performed as soon as possible at the laboratory in the Land of Leopard National Park to reduce chance of DNA degradation, using the identical methods used by Sugimoto et al. (2012, 2013).

Species identification, sex determination, genotyping and population estimation

- Extracted DNA will be transferred to the laboratory of CGRB, Seoul National University, South Korea and species identification, sex determination and genotyping by molecular genetic analysis will be performed. International transfer of DNA extracted from feces will not require CITES permit.
- All the genetic analyses and population estimation will be done using the same methods by Sugimoto et al. (2012, 2013) and Rozhnov et al. (2013). For individual identification of leopards, the identical 13 markers used by Sugimoto et al. (2013) will be utilized.
- Additional markers for leopards will be developed by Genome Research Foundation (Dr. Jong Bhak).
- Species identification and sex determination will be done following methods by Sugimoto et al. (2006, 2012, 2013).
- Transboundary movement of individual animals will be analyzed by tracking the sample sites of individuals found both in China and Russia.

Timeline

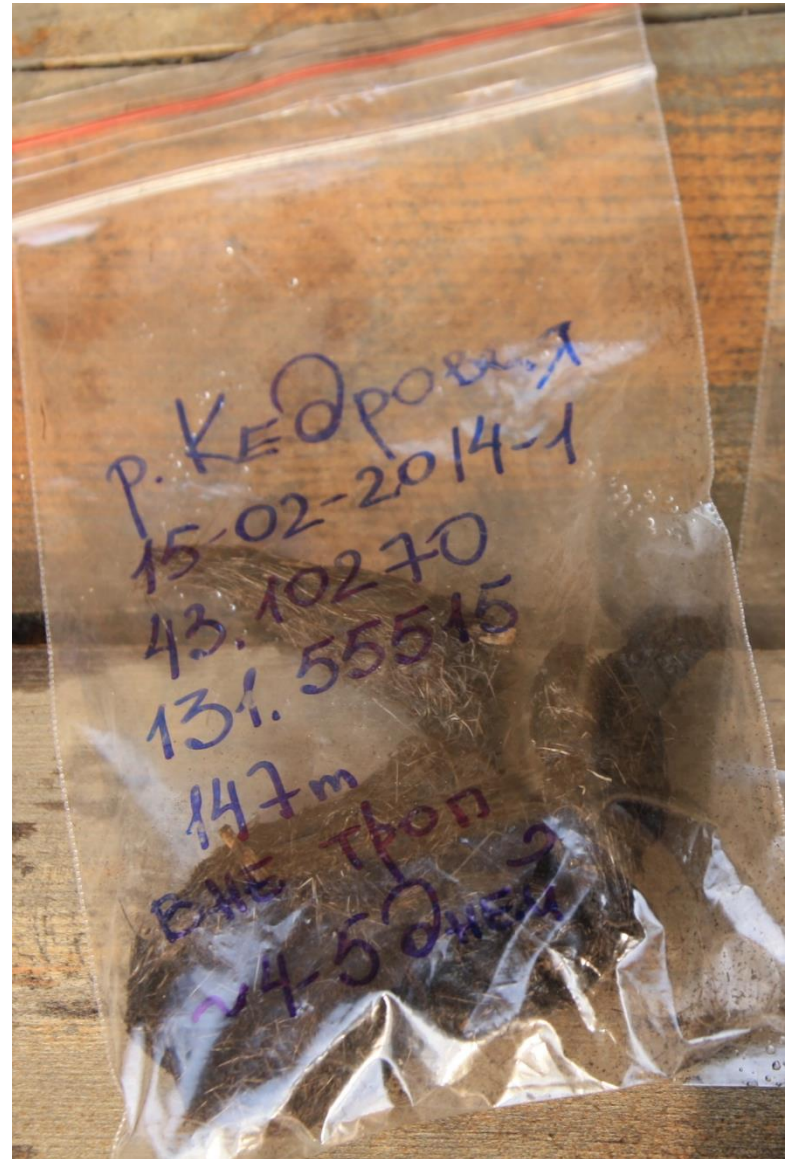
- February-March 2014: Collect samples and extract DNA.
- April-December 2014: Analyze existing samples, develop additional markers, optimize analytical techniques, build collaboration networks. Write preliminary report.
- January-March 2015: Collect samples and extract DNA.
- April-November 2015: DNA and data analysis and final report submission.

Studies ongoing by CGRB, Seoul National University

- Collected tiger and leopard fecal samples from the field of 'Land of the Leopard National Park' in February-March, 2014.
- Extracted DNA from the field and storage.
- Less than 10% of the samples produced DNA concentration lower than 5 ng/ μ g.
- Species identification result (Total 93 samples)
 - Amur Tiger: 59
 - Amur Leopard: 32
 - Unknown: 2

Collection of Fecal Samples





Genotyping

- For individual identification, multiplex PCR for 13 microsatellite markers are being tested according to report by Sugimoto et al., 2013.
- Markers being tested

	MARKER
1	FCA008, FCA026, FCA098
2	FCA097, FCA211, FCA224
3	FCA043, FCA105
4	FCA090, FCA123
5	FCA229, FCA247
6	FCA096

- Additional marker development

Topics

- Sample collection
- Budget
- Coordinating collaboration network