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Molecular Diversity and Phylogeography of the Asian Leopard Cat, *Felis bengalensis*, Inferred from Mitochondrial and Y-Chromosomal DNA Sequences

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To investigate genetic diversity and phylogeography of the Asian leopard cat (*Felis bengalensis*), mitochondrial DNA (mtDNA) sequences were determined for 39 individuals from various areas. Sequences combining the complete cytochrome *b* gene (1,140 bp) with the partial control region (646–810 bp) were classified into 24 haplotypes: 21 types from 21 animals, one from eight animals from Tsushima Islands, one from eight animals from Iriomote Island, and one from two animals from Southeast Asia. Phylogenetic trees of the 24 haplotypes clearly showed three clades: a Northern Lineage and Southern Lineages 1 and 2. The Northern Lineage consisted of animals from Tsushima Islands, the Korean Peninsula, the continental Far East, Taiwan, and Iriomote Island. Within the Northern Lineage, genetic contacts could have occurred between geographically neighboring populations before isolation by straits. Southern Lineage 1, comprising Southeast Asian animals, showed higher genetic diversity. Southern Lineage 2 had large genetic distances from other lineages. Within the control region, the Asian leopard cats shared two to four repetitive motifs, and the number of motifs and their constitution were highly variable among individuals. The motifs were polymorphic even within individuals and could be classified into 31 types. Finally, males of mtDNA Southern Lineage 1 had either of two types of the Y-chromosomal gene *ZFY*, whereas all males of Northern Lineage shared only one type. Our results indicate that the diversity of southern populations is higher and that genetic differentiation among northern local populations reflects past geographical isolation.

Key words: *Felis bengalensis*, Asian leopard cat, mitochondrial DNA, repetitive sequence, *ZFY*

INTRODUCTION

The Asian leopard cat (*Felis bengalensis*) is one of the most widespread species of the family Felidae in Asia. It occurs in forests from South Asia through East Asia to the Russian Far East, and from Southeast Asia to western Indonesia and the Philippines (Nowell and Jackson, 1996). In the Japanese islands, two insular populations of this species are known. One is on Tsushima Islands (696 km^2) located between Japan and the Korean Peninsula (see Fig. 1). Based on morphological analysis, the Tsushima leopard cat has been included with the Far-Eastern populations, known as the Amur leopard cat, which were classified as a subspecies, *F. b. euptilura* (Imaizumi, 1960). Another subspecies is the Iriomote leopard cat from Iriomote Island (289 km^2) located approximately 200 km east of Taiwan (see Fig. 1). Population sizes of both the Tsushima and Iriomote leopard

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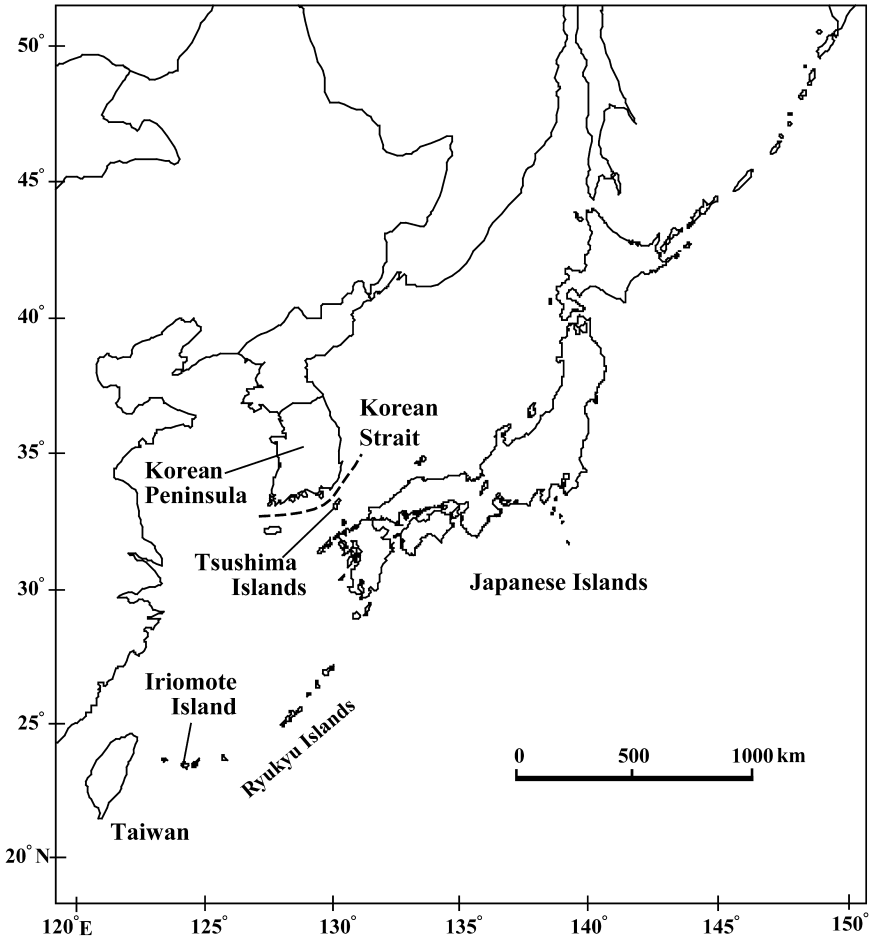

Fig. 1. Sampling localities for the Asian leopard cat in northeastern Asia. In the present study, we found this area to be occupied by the Northern Lineage of mtDNA (see Table 1 and the text).

cats were estimated at approximately 100 on each island (Izawa and Doi, 1991; Izawa et al., 1991). The Tsushima leopard cat has been protected as a Natural Monument since 1971, and the Iriomote leopard cat has been protected as a Special Natural Monument since 1972. The genetic diversities of the Tsushima and Iriomote leopard cat populations have decreased due to bottlenecks and/or inbreeding caused by geographic isolation on the islands (Masuda and Yoshida, 1995). For conservation and management of these endangered feline populations, there is an urgent need to understand their phylogenetic status and genetic characteristics.

Morphological characters of the Asian leopard cats are highly variable, and differences have been reported mainly between groups of specimens obtained from northern and southern areas of Asia (Kitchener, 1991). Generally, the Asian leopard cats of northern Asia have shorter tails, longer bodies, and shorter legs than those of southern Asia (Kitchener, 1991). The coloration of Asian leopard cats tends to be more yellowish-brown in the tropics and more grayish-brown in the northern parts of their range (Nowell and Jackson, 1996). Imaizumi (1967) examined morphological characters of the Iriomote leopard cat and classified it as a new genus and new species, *Mayailurus iriomotensis*. Glass and Todd (1977) reported morphological variation in Asian leopard cats in the presence or absence of the second upper premolar, and positioned the Iriomote population into a population of *F. bengalensis*.

Wurster-Hill et al. (1987) reported that the G-banding karyotype of the Iriomote Asian cat (2n=38) was indistinguishable from that of continental populations of the Asian leopard cat. Suzuki et al. (1994) examined restriction sites in nuclear ribosomal genes between the Iriomote and continental leopard cats, and found no differences between them. Masuda et al. (1994) and Masuda and Yoshida (1995) analyzed 402 bp of the mitochondrial cytochrome *b* gene and showed that both the Iriomote and Tsushima leopard cats are closely related to the continental Asian leopard cats. Furthermore, analysis of partial sequences of four mitochondrial genes (NADH dehydrogenase subunit 5, ATPase-8, 16S ribosomal RNA, and cytochrome *b*) as well as allelic variation at 18 nuclear microsatellite loci showed that the Iriomote leopard cat is closely related to the Asian leopard cat from the continent (Johnson et al., 1999). Based on the molecular phylogeny and the previously reported morphological data, Masuda and Yoshida (1995) supported classification of the Iriomote and Tsushima leopard cats as subspecies *F. b. iriomotensis* and *F. b. euptilura*, respectively. However, the genetic variability among Asian leopard cat populations across their distribution ranges has not been fully studied.

In the present study, in order to further understand the genetic diversity and phylogeography of the Asian leopard cats, we determined 1) the complete sequences of the maternally inherited mitochondrial cytochrome *b* gene (1,140 bp), 2) partial sequences of the mtDNA control region (646–810 bp), and 3) partial sequences (666 bp) of the final intron of *ZFY* (zinc-finger gene on the Y chromosome) for Asian leopard cats from Southeast Asia, the continental Far East, Taiwan, Korea, and Iriomote and Tsushima Islands of Japan. Because the noncoding sequence of the large intron within the *ZFY* gene shows more rapid evolution than the coding sequences of the exons, it has become an invaluable marker for determining paternal phylogenetic relationships among felid species (Pecon-Slattery and O'Brien, 1998; Pecon-Slattery et al., 2000; Pecon-Slattery et al., 2004). Based on comparisons of maternally and paternally inherited DNA sequences, we discuss the molecular diversity, phylogeography and migration history of Asian leopard cat populations.

MATERIALS AND METHODS

Samples and DNA extraction

Asian leopard cat tissue samples (blood, muscle, feces or hair) were obtained from 39 animals originating from various areas in Asia (Table 1 and Fig. 1). Total DNA was extracted from blood or tissue samples using the DNeasy Tissue kit (Qiagen). Fecal DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen). An aliquot (1–5 μl) of the DNA extract was amplified by subsequent polymerase chain reaction (PCR). For DNA extraction from hair samples, hair roots approximately 5 mm long were collected, washed with 70% ethanol, incubated overnight in 5% Chelex-100 (Bio-Rad) at 56°C, and then boiled for 8 min (Walsh et al., 1991), and 10 μl of the supernatant was used as the PCR template. The fishing cat *Felis viverrinus*, which is closely related to the Asian leopard cat (Masuda et al., 1996), was used as the outgroup. Eight Tsushima leopard cats (individual nos. TSU1–8, Table 1) examined in the present study were the same as those reported in Tamada et al. (2005), and their complete cytochrome *b* sequence (accession no. AB194818) and part (373 bp) of the control region were reported by Tamada et al. (2005). Part (402 bp) of the cytochrome *b* gene was sequenced for two Asian leopard cats (OKI1 and OKI2) from the continent by Masuda et al. (1994).

PCR conditions and PCR product purification of mtDNA and *ZFY*

To amplify the complete sequence (1,140 bp) of the cytochrome *b* gene, primer set Cb-M1/Cb-MR2 was used for first-round PCR, and Cb-M2/Cb-MR1 for nested PCR (Matsuhashi et al., 1999; Kurose et al., 2001; Tamada et al., 2005). To amplify part of the control region (646–810 bp), the following primer sets were used: FCB-Z/FDL1R (Tamada et al., 2005) or FDL2R (5'-ACG CAA ACG TGG GTG CGC GC-3') for first-round PCR, and FCB-Z/FDL1R for semi-nested PCR. We designed primer FDL2R based on the domestic cat mtDNA sequence reported by Lopez et al. (1996). The primer position of FCB-Z (Tamada et al., 2005) refers to that of primer L15774 reported by Shields and Kocher (1991).

Table 1. Profiles of samples examined in the present study.

Population	Individual no. of animal	Sex	Tissue	Source; Collecting locality if known/Supplier	Haplotype/Accession No.* cytochrome *b*	Haplotype/Accession No.* control region	*ZFY* type of male
Tsushima leopard cat	TSU 1–4**	male	muscle	Tsushima Islands, Japan/Tsushima Wildl. Conserv. Ctr.	FCN1/AB194818**	FDN1/AB210241[&]	ZN1
	TSU 5–8**	female	muscle	Tsushima Islands, Japan/Tsushima Wildl. Conserv. Ctr.	FCN1/AB194818**	FDN1/AB210241[&]	
Iriomote leopard cat	IRI 1–4	male	muscle	Iriomote Island, Japan/Iriomote Wildl. Conserv. Ctr.	FCN4/AB210228	FDN6/AB210246	ZN1
	IRI 5–8	female	muscle	Iriomote Island, Japan/Iriomote Wildl. Conserv. Ctr.	FCN4/AB210228	FDN6/AB210246	
Taiwan leopard cat	TWA 1	female	hair	Taiwan/Taiwan Tunghai University	FCN3/AB210227	FDN7/AB210247	
Continental leopard cat	ING 1	male	feces	Korean Peninsula/Inokashira Park Zoo, Japan	FCN1/AB194818#	FDN5/AB210245	ZN1
	ING 2	male	feces	Korean Peninsula/Inokashira Park Zoo, Japan	FCN1/AB194818#	FDN4/AB210244	ZN1
	ING 3	female	feces	Korean Peninsula/Inokashira Park Zoo, Japan	FCN1/AB194818#	FDN3/AB210243	
	ITO 1	male	muscle	Continental Far East/Itozu Zoo, Japan	FCN2/AB210226	FDN2/AB210242	ZN1
	UEN 1	male	blood	Thailand/Ueno Zoological Gardens, Japan	FCS2/AB210230	FDS11/AB210258	ZS1
	UEN 2	male	blood	Thailand/Ueno Zoological Gardens, Japan	FCS2/AB210230	FDS8/AB210255	ZS1
	UEN 3–4	female	blood	Southeast Asia/Ueno Zoological Gardens, Japan	FCS9/AB210237	FDS16/AB210263	
	OKI 1[$]	male	blood	Southeast Asia/Okinawa Kids Discovery Kingdom, Japan	FCS2/AB210230	FDS4/AB210251	ZN1
	OKI 2[$]	male	blood	Southeast Asia/Okinawa Kids Discovery Kingdom, Japan	FCS1/AB210229	FDS1/AB210248	ZN1
	AKI 1	female	blood	Southeast Asia/Akita Omoriyama Zoo, Japan	FCS2/AB210230	FDS2/AB210249	
	AKI 2	male	blood	Southeast Asia/Akita Omoriyama Zoo, Japan	FCS2/AB210230	FDS12/AB210259	ZS1
	FBD1	male	hair	Thailand/Dusit Zoo, Thailand	FCS2/AB210230	FDS3/AB210250	ZN1
	FBD2	male	hair	Thailand/Dusit Zoo, Thailand	FCS4/AB210232	FDS7/AB210254	ZN1
Continental leopard cat	FBK1	female	hair	Thailand/Khao Kheow Open Zoo, Thailand	FCS5/AB210233	FDS15/AB210262	
	FBK2	male	hair	Thailand/Khao Kheow Open Zoo, Thailand	FCS7/AB210235	FDS13/AB210260	ZN1
	FBK3	male	hair	Thailand/Khao Kheow Open Zoo, Thailand	FCS6/AB210234	FDS10/AB210257	ZN1
	CHI1	male	hair	Thailand/Chiangmai Zoo, Thailand	FCS8/AB210236	FDS6/AB210253	ZN1
	CHI2	female	hair	Thailand/Chiangmai Zoo, Thailand	FCS3/AB210231	FDS14/AB210261	
	NEG1	male	hair	Southeast Asia/Zoo Negara, Malaysia; Fukuoka Zoo, Japan	FCS2/AB210230	FDS9/AB210256	ZN1
	NEG3	female	hair	Southeast Asia/Zoo Negara, Malaysia; Fukuoka Zoo, Japan	FCS10/AB210238	FDS17/AB210264	
	HAT1	male	hair	Southern Thailand/Obihiro Univ. of Agr. and Vet. Med.	FCS2/AB210230	FDS5/AB210252	ZN1
Fishing cat (outgroup)	FVDZ1-3		hair	Thailand/Dusit Zoo, Thailand	FVC2/AB210240	FVD2/AB210266	
	FVK1		hair	Thailand/Khao Kheow Open Zoo, Thailand	FVC1/AB210239	FVD1/AB210265	
	FVK2		hair	Thailand/Khao Kheow Open Zoo, Thailand	FVC2/AB210240	FVD2/AB210266	

* Sequence data will appear with accession numbers in the DDBJ nucleotide sequence database.
** Cited from Tamada et al. (2005).
[&] Partial control region sequences (373 bp) of these eight animals were determined by Tamada et al. (2005).
[#] Same sequence as FCN1 of TSU1–8.
[$] Partial cytochrome *b* sequences (402 bp) of these two animals were determined by Masuda et al. (1994).

To amplify partial sequences (666 bp) of the *ZFY* final intron, the following primer sets were used: ZFYC1F (5'-GTG AGG GTG CAC AAG TTC CCA C-3') or ZFYC2F (5'-ACA GTG CAG TGT GCT CTG TG-3')/ZFYC2R (5'-AGA AAA GAA CAT GAG TGA TCA AAC-3') for first-round PCR, and ZFYC2F/ZFYC3R (5'-GAG TGA TCA AAC AAC GGT TTG-3') for semi-nested PCR. We designed these four primers based on the Asian leopard cat *ZFY* final intron sequence (accession no. AY518640) reported by Pecon-Slattery and O'Brien (1998), Pecon-Slattery et al. (2004), and Dr. J. Pecon-Slattery (Personal communication).

The total volume (50 μl) of PCR reaction mixtures contained 1–10 μl of DNA extract, 5 μl of 10 x buffer (Takara), 4 μl of dNTP (2.5 mM of each dNTP, Takara), 1.25 U of r*Taq* DNA polymerase (Takara), and 0.5 μl of each primer (25 pmol/μl). For hair extracts, 20 mg of bovine serum albumin (Boehringer) was applied to the reaction mixture to eliminate the effects of PCR inhibitors, which are often contained in hair extracts. PCR amplifications were carried out in a PCR thermal cycler (TP400, Takara). PCR cycle conditions for cytochrome *b*, the control region, and the *ZFY* intron were one cycle of 94°C, 3 min; 35–40 cycles of 94°C/1 min, 55–60°C/1 min, 72°C/1 min; and one cycle of 72°C, 10 min. When the first PCR amplification failed to produce visible products detected by agarose gel electrophoresis, semi-nested PCR using one of the first-round PCR primers and one inner primer for the opposite site, or nested PCR using two inner primers, was carried out with 30–40 cycles. Bovine serum albumin was not added to the reaction mixtures for nested and semi-nested PCRs. A 10-μl aliquot of the PCR product was electrophoresed on a 2% agarose gel and stained by ethidium bromide, and the DNA band in the gel was then visualized under an ultraviolet illuminator. The remaining PCR products (40 μl) were purified by the QIAquick Centrifugal Dialysis Kit (Qiagen).

Direct sequencing and data analysis

Cycle PCRs were done with the Thermo Sequenase Primer Cycle Sequencing Kit (Amersham) and sequencing primers labeled with Texas Red at the 5'-end position. Sequencing of cycle PCR products was performed with an automated DNA sequencer (Hitachi SQ-5500). Sequencing primers for cytochrome *b* were the same as used by Tamada et al. (2005), and newly designed FBEN2 (5'-CCT CCT CAT ATC AAG CCC G-3') and FBEN3 (5'-ACC TTC TCA GAG ACA TGA AAC-3') were also used. Sequencing primers for the control region were the same as used by Tamada et al. (2005) and newly designed FDL5R (5'-GGT TTC TCG AGG CTA GCT G-3') and FDLSQ5 (5'-ACA TAA GAC ATA TAG TGT TTG GT-3')(Fig. 1) were also used. Sequencing primers for the *ZFY* intron were ZFYC2F and newly designed ZFYIN1F (5'-TGG ACA GTT CCT TTG CAG TAA-3') and ZFYIN2R (5'-ACT GCA AAG GAA CTG TCC A-3').

Sequence alignment was done using the program GeneWorks (Intelligenetics). Positions of insertions or deletions (indels) that appeared in the control region were adjusted by eye and were excluded from subsequent genetic-distance estimation and phylogenetic analyses. Proportion (p) distances calculated by MEGA ver. 3.1 (Kumar et al., 2004) were used for percentage differences and similarities among sequences. The sequence diversity (π) of haplotypes was also calculated in MEGA.

Phylogenetic trees of sequence data combining cytochrome *b* with the control region were constructed by the maximum likelihood (ML), maximum parsimony (ML), and neighbor-joining (NJ: Saitou and Nei 1987) methods implemented in PAUP* ver. 4.0b10 (Swofford, 2001). For the ML method, the hierarchical likelihood ratio test (hLRT) was implemented in Modeltest ver. 3.6 (Posada and Crandall, 1998) to find the nucleotide substitution model that best fit the data. This test selected the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) including invariable sites and rate variation among sites (HKY+I+G). MP trees were obtained using the heuristic search option with random sequence addition and tree-bisection-reconnection (TBR) branch swapping. The NJ tree was constructed using Kimura (1980) two-parameter (K2P) distances. Bootstrap values (Felsenstein, 1985) were obtained from 1,000 pseudoreplicates for both MP and NJ, and 200 pseudoreplicates for ML, to assess the support for internal nodes in the trees. A parsimony network of sequences was constructed using TCS ver. 1.21 (Clement et al., 2000).

RESULTS

MtDNA sequence variations in the Asian leopard cats

From all 39 Asian leopard cats examined, the complete (1,140 bp) cytochrome *b* sequence was determined, and 14 types were identified (FCN1–4 and FCS1–10) (Table 1). Partial sequences (646–811 bp) of the control region were determined, and 24 types (FDN1–7, FDS1–17) were identified (Table 1). The sequence length differences resulted from variation in repetitive sequences, mentioned below. For the phylogenetic analyses, 24 haplotypes (numbered in Table 1) obtained by combining the two genes were used. The eight Tsushuma leopard cats (TSU1–8) shared the same mtDNA sequence. The eight Iriomote leopard cats (IRI1–8) shared another mtDNA sequence, as did two individuals (UEN3 and UEN4) from Southeast Asia (Table 1). The sequences of the other animals were different from individual to individual. Of the five fishing cats used as the outgroup, two types (FVC1 and FVC2) of cytochrome *b* and two types (FVD1 and FVD2) of the control region were obtained (Table 1); these comprised two mtDNA haplotypes based on combined sequences of the two genes (Fig. 2). Accession numbers for the cytochrome *b* and control region sequences obtained in the present study are shown in Table 1.

Molecular phylogenetic relationships among the Asian leopard cats

In the data set for the MP analysis, including the outgroup, there were 161 parsimony-informative sites among the 1,758 bp of the mtDNA haplotypes, excluding indels. In the analysis, the consistency index (CI) was 0.7072 and the retention index (RI) was 0.8292 in the consensus MP tree.

Because the molecular phylogenetic trees constructed by the ML, MP, and NJ methods all indicated that the Asian leopard cats examined group into three clades, we show the ML tree as a representative topology, together with bootstrap values obtained from the three methods (Fig. 2). We termed as the "Northern Lineage" a clade consisting of animals from northern Asia (Tsushima Islands, continental Far East, Korean Peninsula, Iriomote Island and Taiwan) with 95/99/97% (ML/MP/NJ) bootstrap values. Haplotypes from Southeast Asian animals formed two clades that we termed "Southern Lineage 1" and "Southern Lineage 2" (Fig. 2). Southern Lineage 1 consisted of 15 animals and was supported with 72/85/51% bootstrap values. In addition, this lineage excluding three animals (OKI2, FBK1 and FBK3) was supported by 96/98/99% bootstrap values. Southern Lineage 2 was composed of three animals (NEG3, UEN3 and UEN4) and first split from the other lineages with 100/100/100% bootstrap values. Thus, Southern Lineage 1 was more closely related to the Northern Lineage (supported with 100/100/100% bootstrap values) than to Southern Lineage 2 (Fig. 2).

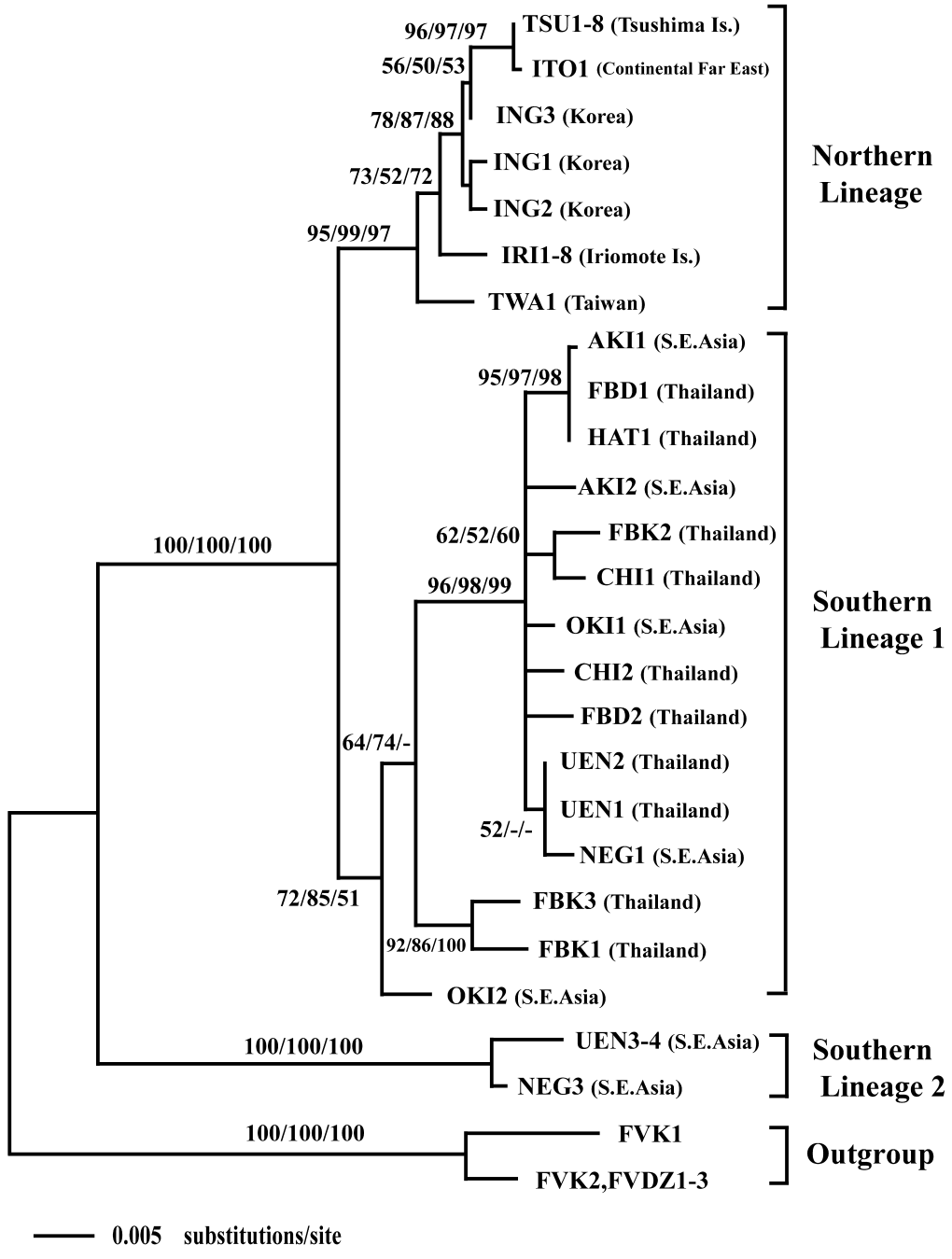

Fig. 2. Phylogenetic relationships among mtDNA haplotypes (sequence data comprising the cytochrome *b* and control region) constructed using the ML method. Bootstrap values obtained by the ML/MP/NJ methods are shown near internal nodes. Three clades were named the Northern Lineage and Southern Lineages 1 and 2. Sequences from fishing cats were used as the outgroup.

Table 2 indicates that the genetic diversity of Southern Lineage 1 is highest among the three lineages. Although the genetic diversity within the Northern Lineage was smaller than that within Southern Lineage 1, animals of the Northern Lineage were genetically differentiated among sampling localities, including islands (Fig. 2).

Repetitive motifs in the control region

Repetitive sequence (RS2) arrays, named according to Lopez et al. (1996), were found in the control region of the Asian leopard cats examined. The arrays were composed of two to four motifs of 80 to 82 bp each. Excluding indels in the repetitive arrays, 23 sites were polymorphic (28.8%; 23/80 sites) among motifs (Table 3). Of the 23 polymorphic sites, mutations at four sites were transversions (A/C at position 58; A/T at positions 66 and 71; C/G at position 60) and those at the other sites were transitions. A total of 33 motifs comprising the arrays were identified from the animals examined. The 5' side motifs were named types A to S and the 3'-most motifs were types a to n, while two motifs

Table 2. MtDNA sequence (combining the cytochrome b with the control region) variability in three lineages of the Asian leopard cats.

Lineage	No. of animals examined	No. of haplotypes	Polymorphic site	Sequence difference between haplotypes, % (average)	Sequence diversity $\pi \pm$ S.E. ($\times 10^{-3}$)
Northern Lineage	21	7	32	0.05 – 1.13 (0.65)	6.48±1.2
Southern Lineage 1	15	15	65	0 – 1.79 (0.92)	9.15±1.1
Southern Lineage 2	3	2	16	0.82	8.22±2.1
All the three lineages	39	24	152	0–5.21 (1.93)	19.33±1.7

Indel sites between haplotypes were eliminated from data caliculation.

Table 3. Variable nucleotide positions among 33 types of repetitive motifs (RS2) in Asian leopard cats and fishing cats treated as the outgroup.

	Variable position*																												
Motif	2	3	4	6	23	25	29	39	40	41	42	43	47	48	53	58	59	60	65	66	67	70	71	73	74	79	82	83	
A	A	T	T	T	T	T	G	C	T	C	A	T	–	–	A	C	A	C	C	T	A	T	A	A	–	G	T	T	
B	.	.	.	C	.	.	.	.	.	.	.	.	–	–	.	.	.	.	.	.	.	.	.	.	–	.	.	.	
C	.	.	.	C	.	.	.	T	.	.	.	.	–	–	.	.	.	.	.	.	.	.	.	.	–	.	.	.	
D	.	.	.	C	.	.	.	T	C	T	.	.	–	–	.	.	.	.	.	.	.	.	.	.	–	.	.	.	
E	.	C	.	C	.	.	.	.	C	T	.	.	T	A	.	.	.	.	.	.	.	.	.	.	–	.	.	.	
F	.	C	.	C	.	.	.	.	C	T	.	.	–	–	.	.	.	.	.	.	.	.	.	.	–	.	.	.	
G	.	C	.	C	.	.	.	T	C	T	.	.	–	–	.	.	.	.	.	.	.	.	.	.	–	.	.	.	
H	.	C	.	C	C	.	.	.	C	T	.	.	–	–	.	.	.	.	.	.	.	.	.	.	–	.	.	.	
I	.	C	.	C	.	.	.	.	C	T	.	.	–	–	.	A	.	.	.	.	.	.	.	.	–	.	.	.	
J	.	C	.	C	.	.	.	T	C	T	.	.	–	–	.	A	.	.	.	.	.	.	.	.	–	.	.	.	
K	.	C	.	C	.	.	.	T	C	T	.	C	T	A	.	.	.	.	.	.	.	.	.	.	–	.	.	.	
L	G	C	.	C	.	.	.	T	C	T	.	.	T	A	.	.	.	.	.	.	.	.	.	.	–	.	.	.	
M	.	C	.	C	.	.	.	T	C	T	G	.	–	–	.	.	.	.	.	.	.	.	.	.	–	.	.	.	
N	.	C	.	C	.	.	.	T	C	T	.	.	–	–	G	.	.	.	.	.	.	.	.	.	–	.	.	.	
O	.	C	.	C	.	.	.	T	C	T	.	C	–	–	.	A	.	.	.	.	.	.	.	.	–	.	.	.	
P**	.	.	C	C	.	G	.	T	C	T	.	.	–	–	.	A	.	.	.	.	.	.	.	.	–	.	.	.	
Q	.	C	.	C	.	.	.	T	C	T	.	.	–	–	G	T	.	.	.	.	.	.	.	.	–	.	.	.	
R	.	C	.	C	.	.	.	T	C	T	.	C	–	–	.	A	.	.	T	.	.	.	.	.	–	.	.	.	
S	.	C	.	C	.	.	A	T	C	T	.	.	–	–	G	T	.	.	.	.	.	.	.	.	–	.	.	.	
a	.	.	.	.	.	.	.	T	.	.	.	C	–	–	.	A	G	A	T	A	G	.	T	G	G	.	.	.	
b	.	.	.	C	.	.	.	T	.	.	.	.	–	–	.	A	G	A	T	A	G	.	T	G	G	.	.	.	
c	.	.	.	C	.	.	.	T	.	.	.	.	–	–	.	A	G	A	.	A	G	.	T	G	G	.	C	.	
d	.	.	.	C	.	.	.	T	.	.	.	.	–	–	.	A	G	A	T	A	G	C	T	G	G	.	.	.	
e	.	C	.	C	.	.	.	T	C	T	.	.	–	–	.	A	G	A	T	A	G	.	T	G	G	.	.	.	
f	.	.	.	C	.	.	.	T	C	T	.	.	–	–	.	A	G	A	T	A	G	.	T	G	G	A	.	.	
g	.	C	.	C	.	.	.	T	C	T	.	.	–	–	.	A	G	A	T	A	G	C	T	G	G	.	.	.	
h	.	.	.	C	.	.	.	T	C	T	.	.	–	–	.	A	G	A	T	A	G	.	T	G	G	A	.	C	
i	.	C	.	C	.	.	.	T	C	T	.	.	–	–	.	A	G	G	T	A	G	C	T	G	G	.	.	C	
j	.	C	.	C	.	.	.	T	C	T	.	.	–	–	.	A	G	A	T	A	G	C	T	G	G	.	.	C	
k	.	C	.	C	.	.	.	T	C	T	.	.	–	–	.	A	G	A	T	A	G	.	T	G	G	A	.	C	
l	.	C	.	C	C	.	.	T	C	T	.	.	–	–	.	A	G	A	T	A	G	.	T	G	G	.	.	C	
m**	.	.	C	C	.	G	.	T	C	T	.	.	–	–	.	A	G	A	T	A	G	.	T	G	G	.	.	C	
n	.	C	.	C	.	.	.	T	C	T	.	.	–	–	.	A	G	A	T	A	G	C	T	G	G	A	.	A	

* Dots indicate identity with the nucleotides present in the A-type motif. Dashes show deletions.

** Types identified from the fishing cats.

(P and m) were from the fishing cats as the outgroup (Tables 3 and 4). Percentage sequence differences were 0–20% (average 9.2%) among the 31 motifs of the Asian leopard cats, and 3.8–18.8% between motifs of the Asian leopard cats and the fishing cats.

In animals classified into the Northern Lineage (Fig. 2), the eight Tsushima leopard cats (TSU1–8) shared four motifs (A/A/A/a) and eight Iriomote leopard cats (IRI1–8) shared three (C/C/c) (Table 4). Three Korean leopard cats had three (B/B/b in ING2) or four motifs (B/B/B/b in ING1 and ING3), and the continental Far Eastern leopard cat (ITO1) had three (A/A/a). The Taiwanese leopard cat (TWA1) had three motifs (C/C/d). By contrast, animals of Southern Lineage 1 shared not only three or four motifs but also two motifs. The three animals (UEN3, UEN4 and NEG3) of Southern Lineage 2 had four motifs (I/I/J/g and O/O/R/i). Thus, numbers, orders and sequences of the repetitive motifs were highly variable in the Asian leopard cats (Table 4).

In a parsimony network (Fig. 3) the 33 motifs including

Table 4. Composition of repetitive motifs (RS2) in the mtDNA control region of Asian leopard cats.

Lineage	Individual no.	Repetitive motifs 1st	2nd	3rd	4th	Similarity (%) within individuals
Northern	TSU1–8	A	A	A	a	87.5–100
Lineage	ING1, 3	B	B	B	b	88.8–100
	ITO1	A	A	-	a	87.5–100
	ING2	B	B	-	b	88.8–100
	IRI1–8	C	C	–	c	90–100
	TWA1	C	C	–	d	88.8–100
Southern	AKI1	E	E	G	e	88.8–100
Lineage 1	AKI2	F	F	F	n	85–100
	FBK2	H	H	H	l	87.5–100
	FBD2, HAT1	G	G	–	e	90–100
	FBK3	D	D	–	h	87.5–100
	UEN2	N	N	–	g	87.5–100
	CHI1	L	G	–	k	86.3–98.75
	CHI2	M	Q	–	j	86.3–96.25
	OKI1	K	S	–	g	86.3–95
	FBD1	G	–	–	e	90
	FBK1	N	–	–	k	86.3
	UEN1	N	–	–	g	87.5
	NEG1	N	–	–	e	88.8
	OKI2	D	–	–	f	88.8
Southern	UEN3–4	I	I	J	g	88.8–100
Lineage 2	NEG3	O	O	R	i	87.5–100
Outgroup (Fishing cat)	FVDZ1-3, FVK1-2	P	P	P	m	90–100

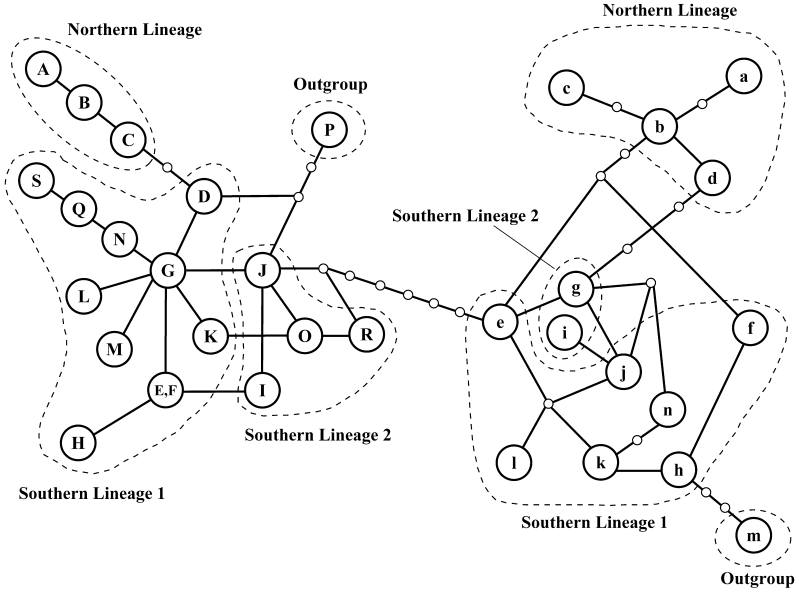

Fig. 3. Parsimony network of repetitive motifs identified in the mtDNA control region. Types A–S are the 5'-side motifs and types a–n are the 3'-most motifs (see Table 3). Small, open circles indicate hypothetical types. Each branch between two motifs means one nucleotide substitution. The Northern Lineage and Southern Lineages 1 and 2 correspond to the same lineages shown in Fig. 2.

outgroup clustered into two groups: the 5'-side motif group (A to S) and the 3'-most ones (a to n). This result indicates that the 3'-most motifs were genetically differentiated from the 5' side ones. Each group was further divided into three sub-groups, which corresponded to the three lineages (Northern Lineage and Southern Lineages 1 and 2). Among the A- to S-types, three motifs (A-, B- and C-types) from the Northern Lineage formed a sub-group (Fig. 3). Southern Lineage 1 was composed of 11 types (D-, E-, F-, G-, H-, K-, L-, M-, N-, Q- and S-types), and the G-type was likely ances-

tral among them. The E- and F-types became the same operational taxonomic unit (OUT) when indels were eliminated. Southern Lineage 2 comprised four motifs (I-, J-, O- and R-types), and the J- or O-type was likely ancestral among them. The P-type of the fishing cats as the outgroup differed in three nucleotides from the D-type of Southern Lineage 1 and the J-type of Southern Lineage 2, indicating that the genetic distances of motifs within the ingroup are larger than those between the ingroup and outgroup. On the other hand, in the phylogenetic relationships among the a- to n-types of the 3'-most motifs, the sub-grouping of motifs from Southern Lineage 2 was unclear, although four motifs (a-, b-, c-, and d-types) of the Northern Lineage formed one sub-group (Fig. 3). This indicates that sequences of the 3'-most motifs are more conserved to each other than those of the 5'-side motifs (Table 3).

Geographical variation of the *ZFY* final intron sequences

Partial sequences (666 bp) of the *ZFY* final intron were determined for 23 male Asian leopard cats. Of the 666 bp, one variable site was observed (T or C at position 548), resulting in classification into two haplotypes: ZN1 (with 'T') and ZS1 (with 'C') (Table 1). Eleven male Asian leopard cats (TSU1–4, IRI1–4, ING1–2, ITO1) from the Northern Lineage shared haplotype ZN1, whereas among 12 males from Southern Lineage 1, nine animals (FBD1-2, FBK2-3, CHI1, NEG1, OKI1-2, HAT1) had ZN1 and three animals (AKI2, UEN1-2) (Table 1) had ZS1. No males were included among the animals sampled from Southern Lineage 2.

DISCUSSION

Phylogeography and migration history of the Asian leopard cats

The present study revealed the phylogeography of the Asian leopard cats across wide geographic area, based on both cytochrome *b* and control region sequences (1,786–1,950 bp) as well as sequence data from the *ZFY* final intron (666 bp). Our results clearly show occurrence of at least three lineages of the Asian leopard cat, one in northern Asia (the Northern Lineage) and two in southern Asia (Southern Lineages 1 and 2) (Fig. 2). Johnson et al. (1999) examined a total of 1,119 bp from partial sequences of four mitochondrial genes (NADH dehydrogenase subunit 5, ATPase-8, cytochrome *b* and 16S ribosomal RNA) and reported two main clusters, one consisting of subspecies *bengalensis* in Southern Asia and the other comprising subspecies *euptilura* and *iriomotensis*. However, the exact geographic origins of samples used by Johnson et al. (1999) were unknown. In contrast, in the present study, we investigated more samples from northern Asian leopard cats (including *euptilura* and *iriomotensis*), with clear locality data (Table 1 and Fig. 1). We obtained longer mtDNA sequences (1,786–1,950 bp per individual, including the complete cytochrome *b* sequence and a partial sequence of the control region), a sequence from the *ZFY* final intron (666 bp), and new genetic information on repetitive sequence (RS2) arrays. With these data, we identified Southern Lineage 2 among Southeast Asian animals. Here we discuss the phylogeographical history of the Asian leopard cat across Asia.

Using a substitution rate of 1.38% per million years (Myr) for feline cytochrome *b* (Masuda et al., 1994), we estimate the time of divergence between the Northern Lineage and Southern Lineage 1 (0.5–1.0% sequence differences) at approximately 0.2–0.4 Myr, that between the Northern Lineage and Southern Lineage 2 (3.1–4.0% sequence differences) at approximately 1.1–1.5 Myr, and that between Southern Lineages 1 and 2 (3.6–4.4% sequence differences) at approximately 1.3–1.6 Myr. These dates indicate that the Southern Lineages of the Asian leopard cats diverged in the early Pleistocene. Johnson et al. (2006) reported that the Asian leopard cat, fishing cat, and flat-headed cat (*F. planiceps*) diverged 2.94 Ma, showing radiation history of all felid species. Divergence times within the Asian leopard cat lineage obtained in the present study are not discordant with the data of Johnson et al. (2006). Divergence times within the Northern Lineage (0.1–0.4% sequence differences), within Southern Lineage 1 (0.1–0.8% sequence differences), and within Southern Lineage 2 (0.7% sequence differences) are approximately 0.03–0.13 Myr, 0.03–0.29 Myr, and 0.25 Myr, respectively.

In the Northern Lineage, the Tsushima and Korean leopard cats grouped together with 78/87/88% bootstrap values (Fig. 2), indicating a very young divergence time (<0.1 Myr). We estimate the divergence time between the Tsushima and continental Far Eastern leopard cats (sequence difference 0.09%) to be approximately 0.03 Myr. Ohshima (1990, 1991) reported that the Korean Strait between the Korean Peninsula and Tsushima Islands formed approximately 0.1 Myr ago. It is thus possible that the ancestor of the Tsushima population had immigrated to Tsushima from the Korean Peninsula by the time of the last formation of the Korean Strait (Fig. 1).

The divergence (0.3% sequence difference) between the Iriomote and the Taiwan leopard cats was estimated to have occurred approximately 0.09 Myr ago. This dating is supported by the formation date (0.24–0.02 Myr ago) of the Kerama Saddle, located in the eastern margin of the Okinawa Trough around the Ryukyu Islands (Kimura et al., 1992; see Fig. 1). Moreover, similarity in the constitution of repetitive arrays between the Iriomote (C/C/c) and Taiwan wild cats (C/C/d) (Table 4) suggests that genetic contacts could have occurred between their ancestors before a strait isolated the two populations geographically, supporting the opinion of Masuda et al. (1994).

MtDNA data comprising the control region and cytochrome *b* reveals a high degree of genetic differentiations among the Asian leopard cats. However, no intra-populational sequence differences were observed in either the Tsushima or Iriomote leopard cats, indicating that genetic diversity has been extremely reduced in these populations compared with other populations of the Asian leopard cat. Masuda and Yoshida (1995) reported that the lack of variation in the two Japanese (Tsushima and Iriomote) populations could have resulted from genetic drift by geographical isolation for approximately 100,000 years on Tsushima Islands and for approximately 200,000 years on Iriomote Island.

As shown in Table 2, Southern Lineage 1 showed the highest degree of genetic variation in the number of haplotypes, the number of polymorphic sites, sequence differences, and sequence diversity (π) among the three lineages identified. In addition, Southern Lineage 2 was identified from animals of Southeastern Asia. Furthermore, the

present study revealed that there are two types (ZN1 and ZS1) of the *ZFY* gene in the Asian leopard cats (Table 1). All males from the Northern Lineage of mtDNA had haplotype ZN1. In contrast, males from Southern Lineage 1 had either ZN1 or ZS1. Identification of this higher level of genetic variation in mtDNA and *ZFY* from southern Asian populations indicates that the origin of the currently widespread Asian leopard cat was somewhere in southern Asia.

We consider two hypotheses to explain the formation of the Northern and Southern Lineages of mtDNA. One is that ancestral populations in both northern and southern areas included polyphyletic mtDNA haplotypes, and that due to genetic drift through bottleneck(s), monophyletic lineages were fixed separately in northern and southern areas of Asia. Some environmental change may have caused the bottlenecks in the ancestral populations. The other explanation is that one monophyletic ancestral lineage was separated from other lineages by some geographical barrier, resulting in no gene flow between populations currently living in northern and southern areas of Asia. The estimated divergence time between the Northern Lineage and Southern Lineage 1 is approximately 0.2–0.4 Myr, as mentioned above. During this period, each of the isolated populations has separately accumulated genetic mutations. In the future, it will be necessary to clarify what serves as a geographical barrier between these lineages, and where it is located.

Southern Lineage 2 was composed of three animals (UEN3, UEN4 and NEG3) and was extremely different (3.1–4.4% for the cytochrome *b*; 5.2–7.7% for the control region) from the Northern Lineage and Southern Lineage 1 (Fig. 2). These values are similar to genetic distances between the Asian leopard cat and the fishing cat. Considering the genetic distances and phylogenetic relationships, Southern Lineage 2 could have been genetically isolated somewhere in Southeast Asia since the early Pleistocene. This lineage may be regarded as an 'evolutionarily significant unit' (Ryder, 1986), as reported in a South American feline species, the margey, *Leopardus wiedii* (Eizirik et al., 1998). Because the three animals were females, they provided no information on *ZFY* types. In addition, exact information on the localities of origin of these zoo-kept individuals (UEN3, UEN4 and NEG3, Table 1) was unfortunately not available. To our knowledge, no morphological differences have been documented between animals of Southern Lineages 1 and 2. To further reveal the genetic features of these lineages, it will be necessary to investigate animals from a more extensive area of southern Asia.

Characteristics of the repetitive array in the control region of the Asian leopard cat

Lopez et al. (1996) reported that the domestic cat mtDNA control region included two distinct repetitive sequences, RS2 and RS3. In addition, Kim et al. (2001) showed that arrays of these two repetitive sequences occur also in great cats such as species of *Panthera*. The RS2 element consists of three complete 80- to 82-bp motifs that are highly conserved with each other and hypermutable compared with the other portion of the control region. RS3 contains 6- to 8-bp motifs that imperfectly repeat 37 times in the domestic cat (Lopez et al., 1996; Kim et al., 2001). Although we did not determine RS3 sequences for the Asian leopard cats, we demonstrated high variability in RS2 among them for the first time. The sequence similarities (80–100%, Table 3) among the 31 motifs identified were close to those (91–98%) reported by Lopez et al. (1996) for domestic cats. The Asian leopard cats we examined had two to four motifs (Table 4). The 3'-most motif of the domestic cat (RS2c) showed greatest divergence at its 3' end (Lopez et al., 1996). Similarly, in the Asian leopard cats examined in the present study, the 3'-most motifs (a- to n-types) exhibited higher variation at their 3' end (Table 3).

Sequence differences among the 31 motifs of the Asian leopard cats were much higher (0–20%) than those (0–7.3%) of other parts of the control region. The J-type was, however, closely related not only to I-, O-, R-types within Southern Lineage 2 (1.3–3.8%), but also to P-type of the fishing cat (3.8%) (Table 3, Fig. 3). Thus, the repetitive motifs of RS2 were extremely polymorphic within the Asian leopard cats, while conserved between the Asian leopard cat and the fishing cat. It is, however, still unclear why more variation was observed within this species than between species. Similar repetitive sequences in the mtDNA control region have been found in other mammals, including sika deer (about 40 bp of motifs) (Nagata et al., 1999), monkey (Hayasaka et al., 1991), shrew (Stewart and Baker, 1994), bat (Wilkinson and Chapman, 1991), and pig (Ghivizzani et al., 1993). The mechanisms of formation of such repetitive sequences are considered to include slippage and other mutations occurring in DNA replication (reviewed by Jakobsen et al., 1996; Lunt et al., 1998).

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