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Two Japanese Wildcats, the Tsushima Cat and the Iriomote Cat, Show the Same Mitochondrial DNA Lineage as the Leopard Cat *Felis bengalensis*

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ABSTRACT—We previously revealed, based on mitochondrial DNA sequence analysis, that the Iriomote cat is very closely related to the leopard cat *Felis bengalensis*, which is widespread in Asia [24]. In this study, in order to understand the phylogenetic status of the Tsushima cat which is the other wildcat in Japan, partial sequences (402 bases) of the mitochondrial cytochrome b region were determined and compared with those of the Iriomote cat and other feline species. The phylogenetic tree of the cytochrome b sequences indicated that the Tsushima cat and the Iriomote cat have the same mitochondrial DNA lineage as the leopard cat. One or two transitional substitutions were observed among the two Japanese wildcats and the leopard cat. The divergence time (approximately 100,000 years ago) of the Tsushima cat and the leopard cat, estimated by sequence data, was in concordance with the formation date of the Tsushima Island. These results suggest that genetic drift after geographic isolation has brought fixation of some genetic and morphological characters to the Tsushima cat and the Iriomote cat, while these two Japanese wildcats are still genetically close to the continental leopard cat. Considering morphological differences and molecular phylogeny, it is reasonable for the two Japanese wildcats to be classified as two subspecies of *F. bengalensis*.

INTRODUCTION

In Japan, two wildcats, the Iriomote cat and the Tsushima cat, live on the Iriomote Island and the Tsushima Island, respectively. Their population sizes were estimated at about 100 on each island [14, 15], and they have been protected as a Special Natural Monument since 1972 and a Natural Monument since 1971, respectively. For their conservation and management, it is now strongly needed to understand the phylogenetic status and genetic characters of these endangered wildcats.

The Iriomote cat was discovered in 1960, and classified as a new species *Mayailurus iriomotensis*, based on peculiar cranial characters: the ventral border of paroccipital process separated from the auditory bulla; the oval disc on basisphenoid and basioccipital region; the postero-external corner of the third upper premolar evenly rounded without cingulum cusp [12]. In addition, this wildcat has a shorter tail, longer body, and shorter legs than the southern Asian leopard cat *Felis* (or *Prionailurus*) *bengalensis* Kerr, 1792 [16]. The Iriomote cat is brown and covered with dark spots, while the typical leopard cat is yellowish with many black spots [16]. Although subsequent taxonomists [7, 21, 26] disagreed to recognizing the new genus *Mayailurus*, they considered the Iriomote cat as a valid species *Felis* (or *Prionailurus*) *iriomotensis*. By contrast, Glass and Todd [5] reported that the Iriomote cat's key characters showed by Imaizumi [12] are

polymorphic in the leopard cat, and Wozencraft [34] considered the Iriomote cat as a synonym of the leopard cat. To clarify the taxonomic problem, we previously examined molecular phylogeny of mitochondrial DNA (mtDNA) sequences on the Iriomote cat and other feline species, and revealed that this cat is most closely related to the leopard cat in the family Felidae [24]. Suzuki *et al.* [33] reported no difference in restriction sites of nuclear ribosomal DNA between the Iriomote cat and the leopard cat. The close relationship between these two wildcats revealed by molecular analyses was in agreement with the similarity showed by karyological analysis [35].

The Tsushima cat is the other Japanese wildcat, which lives on the Tsushima Island locating between the Tsushima Strait and the Korean Strait. Based on morphological similarity, the Tsushima cat has been included in populations of Far East, which were classified as a subspecies of *F. bengalensis* [4, 11] or a distinct species, the Amur cat *Felis euphilura* Elliot, 1871 [8, 9]. Heptner [8] reported that *F. euphilura* has a larger body and darker or more faintly spotted patterns than *F. bengalensis* from southern Asia, and that the skull is more juvenile in *F. bengalensis*. Gao *et al.* [4], however, compared morphological characters among Asian populations, and considered *euphilura* as one of several subspecies in *F. bengalensis*. The characters of the Tsushima cat are clearly different also from those of the Iriomote cat [12]. Therefore, it is needed to re-examine taxonomy of the form *euphilura*, using the Tsushima cat as a representative of the Far-Eastern wildcats.

In this study, we determined partial sequences of the cytochrome b region for the Tsushima cat and then compared

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them with those of the Iriomote cat, the southern Asian leopard cat, and other feline species. We present the molecular phylogeny of the Tsushima cat, and discuss the taxonomic status of the two Japanese wildcats.

MATERIALS AND METHODS

Sample source and DNA extraction

Profiles of specimens were described in Table 1. Two Tsushima cats (FEU1 and 2) and six Iriomote cats (FIR7-12) were obtained from cases of traffic or other accidents on the Tsushima Island and the Iriomote Island, respectively. These Iriomote cats (FIR7-12) and four leopard cats (FBE3-6) examined in this study were different from individuals used in the previous study [24]. The leopard cats (FBE3-6) were all captured in Southeast Asia and then kept in Japanese zoos (Table 1), although the relationship among them was not clear. Cytochrome b nucleotide sequences of the domestic cat, lynx, snow leopard, and tiger were used from our previous data [24]. The sequence of the suricate [24] from the family Viverridae, which is closer to the family Felidae than other carnivore, was used as an outgroup. Total DNA was extracted from muscle tissues preserved in 70% ethanol, heparinized whole blood or cultured skin fibroblasts, according to the phenol/proteinase K/sodium dodecyl sulfate method of Sambrook *et al.* [30] with some modifications [22–24].

TABLE 1. The Tsushima cats, the Iriomote cats, and the leopard cats examined in this study

Animal	Code	Sample number and source
Tsushima cat	FEU	2 individuals (FEU1, 2) from the Tsushima Island, Nagasaki
Iriomote cat	FIR	6 individuals (FIR7–12) from the Iriomote Island, Okinawa
Leopard cat	FBE	2 captive individuals (FBE3, 4) from Akita Omoriyama Zoo; originated from Southeast Asia 2 captive individuals (FBE5, 6) from Ueno Zoological Gardens; originated from Southeast Asia

PCR amplification and direct sequencing

Following the report of Irwin *et al.* [13], PCR primers for amplification of the cytochrome b region were designed as L14724 (5'-GATATGAAAAACCATCGTTG-3') and H15149 (5'-CTCA-GAATGATATTTGTCCTCA-3') [22–24]. Two other primers, L14721 (5'-GACCAATGATATGAAAAACCATCG-3') and H15151 (5'-GCCCTCAGAATGATATTTGTCCT-3'), were newly synthesized. Primer names identify the light (L) or heavy (H) strand and the 3' end-position of the primer in the human mtDNA sequence [1]. Symmetric and asymmetric PCR methods [6, 19] were performed using *AmpliTaq* DNA polymerase (Perkin Elmer Cetus).

The asymmetric PCR product concentrated by a Centricon-30 microconcentrator (Amicon) was sequenced using T7 DNA polymerase (United States Biochemical) and PCR primers, according to the dideoxynucleotide chain reaction method [31]. The internal primers for sequencing were synthesized as 5'-GACACAACAACCGCCTTCTC-3' (the light strand) and as its complement 5'-GAGA-AGGCGGTTGTTGTGTC-3' (the heavy strand) [24]. Reaction products were electrophoresed on 6% polyacrylamide gels containing

7M urea. Gels were dried and exposed to Fuji RX X-ray films for 1–5 days.

Sequence analysis

Construction of phylogenetic tree by the neighbor-joining method [29] was performed using the Clustal V computer software [10]. Numbers of nucleotide substitution per site were estimated for multiple substitutions using Kimura's two-parameter method [17]. The bootstrap method [3] (1000 replications) was made in the Clustal V to assess the degree of support for internal branching in the phylogenetic tree.

RESULTS AND DISCUSSION

Nucleotide sequences of the cytochrome b region and the phylogenetic tree

For the cytochrome b nucleotide sequences (402 bases) (Fig. 1), no sequence difference was observed between two Tsushima cats (FEU1 and 2). The six Iriomote cats (FIR7–12) newly examined in this study shared the same sequence as six cats (FIR1–6) investigated previously [24]. Sequences of the four leopard cats (FBE3-6) examined here were identical to those of two leopard cats (FBE1 and 2) in the previous study [24].

Between the Tsushima cats (FEU1, 2) and the leopard cats (FBE3-6), one nucleotide substitution (A : G) was observed at the nucleotide number (nt) 354 (Fig. 1, Table 2). The Iriomote cats (FIR7–12) and the leopard cats (FBE3–6) have two substitutions at nt 108 (G : A) and nt 354 (A : G). Between the Tsushima cats (FEU1, 2) and the Iriomote cats (FIR7–12), one substitution (A : G) was observed at nt 108. Thus, nucleotide substitutions among these three wildcats were restricted at two sites, nt 108 and nt 354, in 402 bases of the cytochrome b region, and they were all transitional and synonymous mutations: one codon (CTA or CTG) including nt 108 encodes the leucine and the other codon (ATG or ATA) including nt 354 encodes the methionine (Fig. 1, Table 2). This implies that synonymous mutation does not occur at random through the sequence but does with the bias to certain nucleotide sites.

The sequence differences between the Tsushima cat and the leopard cat, between the Iriomote cat and the leopard cat, and between the Tsushima cat and the Iriomote cat were 0.25% (1/402 bases), 0.5% (2/402 bases), and 0.25% (1/402 bases), respectively. Such degree of the differences was comparable to intraspecific sequence variations of other feline species [24] as well as other carnivores including musteline species [22, 23]. By contrast, more than 10% sequence differences were observed between the Tsushima/Iriomote/leopard cats and the other feline species [24].

Phylogenetic tree of the cytochrome b sequences, constructed by the neighbor-joining method, showed that the Tsushima cat, the Iriomote cat, and the leopard cat were clustered with a high confidence (100% bootstrap value) (Fig. 2). The evolutionary distance among these three wildcats was much smaller than that between two *Panthera* species, the snow leopard (*P. uncia*) and the tiger (*P. tigris*), which

	10	20	30	40	50	60	70	80	90	100	
FEU1,2	ATGACCAACA	TTCGAAAATC	ACACCCCCTT	ATCAAAATCA	TCAACCCTC	ATTCATCGAT	CTACCTGCC	CATCCAACAT	CTCAGCATGA	TGGAACCTCG	
FIR7-12	
FBE3-6	
FLY1,2CT	..T.....C	..G..C...T..	T.....	..A.....	
FCA1T	..T..T....CC.....T.....A.....	
	↓	110	120	130	140	150	160	170	180	190	200
FEU1,2	GCTCCCTACT	AGGAGTATGC	CTAATCTTAC	AAATCCTCAC	CGGCCTTTTC	CTAGCCATAC	ACTACACATC	AGATACAACA	ACCGCCTTCT	CATCAGTCAC	
FIR7-12G..	
FBE3-6A..	
FLY1,2A.T..C	..G.....T.....C.....G..G.	
FCA1T..C...CT..C..T	T.G.....C....T.T.....T..	
	210	220	230	240	250	260	270	280	290	300	
FEU1,2	TCACATCTGT	CGTGACGTTA	ACTACGGCTG	AATCATCCGA	TACATACACG	CCAACGGAGC	CTCCATATTT	TTTATCTGTC	TATACATGCA	CGTAGGTCGA	
FIR7-12	
FBE3-6	
FLY1,2	C..T....C	..C.....GT.CCTA..	
FCA1	C.....	..C.....	..T..T....TT.....	T..T....CC.	..G....A..	T..G..A..G	
	310	320	330	340	350	↓	360	370	380	390	400
FEU1,2	GGAATATATT	ATGGCTCCTA	CACCTTCTCA	GAGACATGAA	ACATIGGAAT	CATACTGCTA	TTTCGAGTCA	TAGCCACAGC	TTTCATGGGT	TACGTACTGC	CA
FIR7-12	
FBE3-6G.....	
FLY1,2C.	..C.....	T.....	..A.....C.....	..C..T.A..	..A...T.	C....A..AC..A..	
FCA1C.	..C.....AT..	..TA.....T....AC..A..	

FIG. 1. Alignment of the cytochrome b nucleotide sequences (402 bases) of the Tsushima cats (FEU1, 2), the Iriomote cats (FIR7-12), and the leopard cats (FBE3-6) determined in this study, together with those of the lynx (FLY) and the domestic cat (FCA) reported in our previous paper [24]. Dots indicate identities with nucleotides in the Tsushima cat sequence. Arrows show two sites, nt 108 and nt 354, of substitutions among the Tsushima cat, the Iriomote cat, and the leopard cat.

TABLE 2. Nucleotide substitutions in the cytochrome b region (402 bases) among the Tsushima cat, the Iriomote cat, and the leopard cat

Animal (Code)	Nucleotide number	
	108	354
Tsushima cat (FEU)	A	A
Iriomote cat (FIR)	G	A
Leopard cat (FBE)	A	G

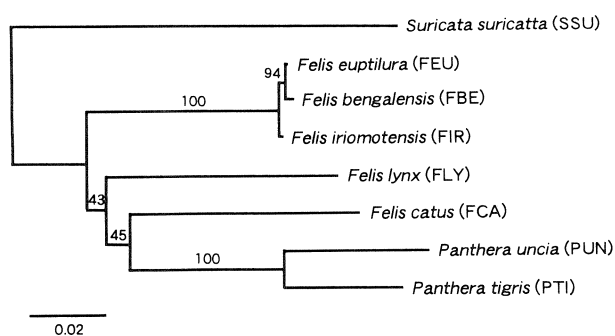


FIG. 2. Neighbor-joining phylogenetic tree of the feline cytochrome b sequences. The scale bar indicates an evolutionary distance of 0.02 substitution per site. Numbers above branches show bootstrap values (%).

were clustered also with 100% bootstrap value (Fig. 2). In addition, the sequence (373 bases) of the 12S rRNA region, which evolves more slowly than the cytochrome b region in

the Felidae [24], was identical among the Tsushima cat, the Iriomote cat, and the leopard cat (data not shown).

Thus, our sequence data indicate that the Tsushima cat and the Iriomote cat have the same mtDNA lineage as the leopard cat. No intra-population sequence difference and small population size (about 100) of each Japanese wildcat [14, 15] suggest that each sequence has been fixed on each island due to genetic drift. No sequence difference among the leopard cats from Southeast Asia may imply a bottleneck in the ancestral population of the Tsushima/Iriomote/leopard cats prior to their divergence, or the close kinship among the leopard cats examined in this study. The cytochrome b sequence data of the Tsushima cat will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D49449.

Divergence time of the Tsushima cat

Based on sequence data of the tiger and the domestic cat, we previously calculated the substitution rate of the feline cytochrome b sequences at 1.38%/million years [24]. Using this value, the divergence of the Iriomote cat and the continental leopard cat was estimated to have occurred about 200,000 years ago [24]. This divergence time was in concordance with the geological isolation date of the Ryukyu Arc including the Iriomote Island [18]. Then, applying this substitution rate to the sequence difference (0.25%) between the Tsushima cat and the leopard cat detected in this study, their divergence time was estimated at about 90,000 years ago. On the other hand, since mammalian cytochrome b

genes were reported to diverge at a rate of at least 2.5%/million years [13, 25], the divergence of the Tsushima cat and the leopard cat was placed to have occurred less than 100,000 years ago. Thus, these two divergence dates estimated by different ways were in good agreement with each other.

Ohshima [27, 28] reported that the Korean Strait and the Tsushima Strait sandwiching the Tsushima Island were geologically formed about 100,000 years ago, when it was in the late Pleistocene. The geological isolation date was in concordance with the divergence time of the Tsushima cat and the leopard cat. The leopard cat is indeed distributed in the Korean Peninsula [2, 11, 20, 26], which faces the Korean Strait on the opposite side of the Tsushima Island.

Controversial classification of the two Japanese wildcats and the continental leopard cat

The leopard cat, which is widespread through southern Asia and eastern Siberia, was first described as *Felis bengalensis* Kerr, 1792. After that, populations from Far East were considered as the Amur cat *Felis euphilura* Elliot, 1871. Heptner [8] reported that the Far-Eastern populations were different from southern Asian populations (*F. bengalensis*) in a larger size, some peculiarities of cranial structure, and type of coloration. Based on the morphological differences, he considered the Far-Eastern populations as a valid species *F. euphilura*, although he could not show the precise border line of distribution of the two taxa [9]. Gao *et al.* [4], however, recognized several subspecies in *F. bengalensis*, and considered *euphilura* as one subspecies. Some taxonomists [2, 20, 26, 34] also did not recognize *euphilura* as a distinct species, but included it in *F. bengalensis*. Schreiber *et al.* [32] reported, based on isozyme analysis, that there was little genetic difference between the Far-Eastern populations and the southern Asian populations. On the other hand, based on the morphological similarity, the Tsushima cat has been included in the Far-Eastern populations, which were classified as *F. euphilura* [9] or as a subspecies of *F. bengalensis* [11]. Therefore, by using the Tsushima cat as a representative of the Far-Eastern populations, we investigated taxonomy of the form *euphilura*.

The cytochrome b sequence difference (0.25%) between the Tsushima cat and the southern Asian leopard cat was quite similar to that (0.5%) between the Iriomote cat and the leopard cat. The magnitude of differences among these three wildcats was comparable to intraspecific variations of other feline species [24] and musteline species [22, 23]. Morphological characteristics, however, is clearly different between the Tsushima cat (or the Far-Eastern populations) and the southern Asian leopard cat. For these reasons, it is reasonable for the Tsushima cat and also the Far-Eastern populations to be considered as a subspecies of the leopard cat *F. b. euphilura*.

On the other hand, it is also the fact that the Iriomote cat shows some morphological characters different from the Tsushima cat or the southern Asian leopard cat. Our molecular analysis, however, revealed the extremely close

relationship among the three wildcats. Suzuki *et al.* [33] reported common restriction patterns of nuclear ribosomal DNA between the Iriomote cat and the eastern Siberian wildcat included in the Far-Eastern populations. Considering morphological characteristics and molecular phylogeny, it is reasonable that the Iriomote cat is classified as a distinct subspecies *F. b. iriomotensis*.

This is the first report of molecular phylogeny of the Tsushima cat, as compared with the Iriomote cat and the continental leopard cat. Such investigation of phylogeny and taxonomy is very important for further study of conservation of the two endangered wildcats in Japan.

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