ZOOLOGICAL SCIENCE 11: 597-604 (1994)

Molecular Phylogenetic Status of the Iriomote Cat *Felis iriomotensis*, Inferred from Mitochondrial DNA Sequence Analysis

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ABSTRACT—To investigate the molecular phylogenetic status of the Iriomote cat *Felis iriomotensis*, partial sequences of the mitochondrial 12S rRNA gene (373 bases) and the cytochrome b gene (402 bases) were determined by using the polymerase chain reaction-product direct sequencing technique and then compared with those of seven other feline species. Six Iriomote cats examined in this study showed no intraspecific variation for both genes. The sequence comparisons and the molecular phylogenetic trees indicated that the Iriomote cat is very closely related to the leopard cat *Felis bengalensis*, which is a widespread species throughout southern and eastern Asia, and that it is reasonable for these two felines to be classified to the same genus. Based on sequence data, the Iriomote cat was estimated to have diverged from the leopard cat arround or less than 0.2 million years ago, and this concurs with the previously reported geological isolation date of the Ryukyu Arc from the Chinese continent. Our results suggest that the geographic barrier has led the fixation of some unique morphological characters into the Iriomote cat population while both the Iriomote cat and the leopard cat still retain very close genetic characters.

INTRODUCTION

The Iriomote cat was discovered in 1965 on Iriomote Island which is located about 200 km east of Taiwan, and Imaizumi [12] examined the morphological characters and classified this feline as a new genus and a new species *Mayailurus iriomotensis*. Since the discovery, some versions of the Felidae classification based on the morphological characters and distribution have been presented as reviewed by Nowak [25]. Although each version recognized the Iriomote cat as a distinct species, the genus nomenclature differed as shown in Table 4. Wurster-Hill *et al.* [36] reported that the G-banding karyotype of the Iriomote cat (2 n =38) was indistinguishable from that of the leopard cat. However, no other analysis on the phylogeny of the Iriomote cat has been reported so far.

On the other hand, the Iriomote cat is one of the most endangered species in Japan. Izawa *et al.* [14, 15] reported that the population of the Iriomote cat was estimated to be about 100 individuals. For the purpose of conservation and management, it is important and also very urgent to clarify the taxonomic position and the genetic characters of this feline.

A molecular genetic approach provides reliable information on the phylogenetic relationships between species. Especially, since the mitochondrial DNA (mtDNA) evolves more rapidly than the nuclear DNA [3], mtDNA sequence analysis is a useful way to study the evolution of closely related species including carnivores [17, 23, 30, 32].

In this study, partial sequences of the mitochondrial 12S rRNA gene and the cytochrome b gene of the Iriomote cat were determined and then compared with those of several other feline species. Based on sequence comparisons, we investigated the phylogenetic status of the Iriomote cat and presented the genetic evidence that this feline is very closely related to the leopard cat, which is a widespread species throughout southern and eastern Asia.

Accepted June 24, 1994
Received March 4, 1994
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MATERIALS AND METHODS

Sample sources and DNA extraction

Tissues specimens were obtained from six Iriomote cats and seven other feline species as described in Table 1. Five Iriomote cats were killed by traffic or other accidents on Iriomote Island [16, 27]. One individual died at the Okinawa Children Land Zoo. Since the family Viverridae and the Hyenidae were reported to be closer to the Felidae than other carnivores [31], the suricate (SSU1) from the Viverridae was used as an outgroup species (Table 1). Species names were according to the nomenclature of Nowak [25]. DNAs were extracted from the frozen muscle, kidney, liver, heparinized whole blood or cultured skin fibroblasts, according to the phenol/proteinase K/sodium dodecyl sulfate (SDS) method of Sambrook *et al.* [28] with some modifications [23].

PCR amplification for single-stranded DNAs

The mitochondrial 12S rRNA and cytochrome b gene regions were PCR-amplified using the procedure of Kocher *et al.* [20] with a slight modification [23]. Primers for the 12S rRNA gene [17], L1091 (5'-AAACTGGGATTAGATACCCCACTAT-3') and H1478 (5'-GAGGGTGACGGGCGGTGTGT-3') were designed by referring to the published sequences of Kocher *et al.* [20]. Referring to the

Table 1. The Iriomote cats and other feline species examined in this study

Species	Common name	Code	Source; Collecting locality if known
Felis iriomotensis	Iriomote cat	FIR	5 individuals (FIR1, 2, 3, 4, 5) stocked at Univ. of Ryukyus; obtained from Iriomote Island, Okinawa
			1 individual (FIR6) kept at Okinawa Children Land Zoo; captured on Iriomote Island
Felis bengalensis	Leopard cat	FBE	2 individuals (FBE1, 2) from Okinawa Children Land Zoo; originated from Thailand (FBE1)
Felis lynx	Lynx	FLY	2 individuals (FLY1, 2) from Asahiyama Zoo, Asahikawa; originated from Siberia
Felis catus	Domestic cat	FCA	2 individuals (FCA1, 2) obtained in Sapporo
Felis pardalis	Ocelot	FPA	1 individual (FPA1) from Asahiyama Zoo
Panthera pardus	Leopard	PPA	2 individuals, PPA1 (studbook no. 173) and PPA2 (studbook no. 161) from Asahiyama Zoo; originated from Siberia
Panthera uncia	Snow leopard	PUN	2 individuals, PUN1 (studbook no. Sapporo 9) and PUN2 (studbook no. Nagoya 4) from Asahiyama Zoo
Panthera tigris	Tiger	PTI	1 individual (PTI1) from Ueno Zoological Gardens; originated from Sumatra
Suricata suricatta	Suricate	SSU	1 individual (SSU1) from Kobe Oji Zoo

report of Irwin *et al.* [13], primers for the cytochrome b gene were designed as L14724 (5′-GATATGAAAAACCATCGTTG-3′) and H15149 (5′-CTCAGAATGATATTTGTCCTCA-3′) [23]. Primer names identify the light (L) or heavy (H) strand and the 3′ end-position of the primer in the human mtDNA sequence [1]. These oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer.

Symmetric PCR was performed with a GeneAmp PCR reagent kit (Perkin-Elmer/Cetus) according to the manufacturer's instruction. In brief, 50 μl of mixture contained 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, each NTP at 200 μM, 1.25 U of *Taq* DNA polymerase, each primer at 250 nM, and 100 ng of the extracted DNA or 1–5 μl of the simply purified DNA as a template. Each cycle consisted of denaturing at 94°C for 1 min, annealing at 50–60°C for 0.5–1 min, and extension at 72°C for 1 min. After 30–35 cycles, the extension reaction was completed by incubation at 72°C for 10 min.

Asymmetric PCR was done to amplify single-stranded DNAs according to the method of Gyllensten and Erlich [9]. The procedures were basically the same as symmetric PCR, except for use of a 1:100 (2.5 nM:250 nM) ratio of the two primers and 1 μl of the symmetric PCR product in 100 μl of the mixture.

Direct sequencing of PCR-amplified single-stranded DNAs

The asymmetric PCR product was concentrated using a Centricon-30 microconcentrator (Amicon), and 7 μl was sequenced with 10 ng of primer for the L or H strand, [^{32}P] dCTP (Amersham), and T7 DNA polymerase (United States Biochemical), according to the dideoxynucleotide chain reaction method [29]. The internal primer for the 12S rRNA sequencing was designed as 5′-GGTTTGCTGAAGATGGCGGTATATAG-3′ (the heavy strand) [17]. For the cytochrome b sequencing, the internal primers were synthesized as 5′-GACACAACAACCGCCTTCTC-3′ (the light strand) and its complement 5′-GAGAAGGCGGTTGTTGTGTC-3′ (the heavy strand). Reaction products were electrophoresed on 6% polyacrylamide gels containing 7 M urea. Gels were dried and exposed to Fuji RX X-ray films for 1–5 days.

Sequence analysis

Sequence alignment and construction of phylogenetic tree by the neighbor-joining method [26] were performed using the Clustal V computer software [11]. Numbers of nucleotide substitutions per site were estimated for multiple substitutions using Kimura's two-parameter method [18]. The bootstrap method [7] was used in the Clustal V to assess the degree of support for internal branches of the phylogenetic tree.

RESULTS

The 12S rRNA sequence of the Iriomote cat

Using the PCR product-direct sequencing technique, partial sequences (373 bases) of the mitochondrial 12S rRNA genes were determined in the Iriomote cat and seven other feline species (Fig. 1). Sequence differences and numbers of transitions/transversions obtained from all pairwise comparisons were shown in Table 2.

The six Iriomote cats (FIR1-6) showed the same sequence (Fig. 1). One (FBE1) of the two leopard cats shared the identical sequence with the Iriomote cats. Between FIR1-6/FBE1 and the other leopard cat (FBE2), there was only one transitional difference (C→T) at the nucleotide position (nt) 301 and the sequence difference was 0.3% (1/373 bases). Higher sequence differences (2.4–4.6%, 9–17/373 bases) were observed between the Iriomote cat and the other feline species (Table 2). For examination of the degree of multiple substitutions, the transversional difference per site was plotted against the transitional difference per site (Fig. 3).

The cytochrome b sequence of the Iriomote cat

Partial sequences (402 bases) of the mitochondrial cytochrome b genes were determined in the Iriomote cats and other feline species. Although the PCR amplification was completed on two specimens (PPA1 and PPA2) of the leopards, the sequencing ladders on the X-ray film were unreadable due to a mixture of more than two kinds of molecule populations, suggesting the heteroplasmy of

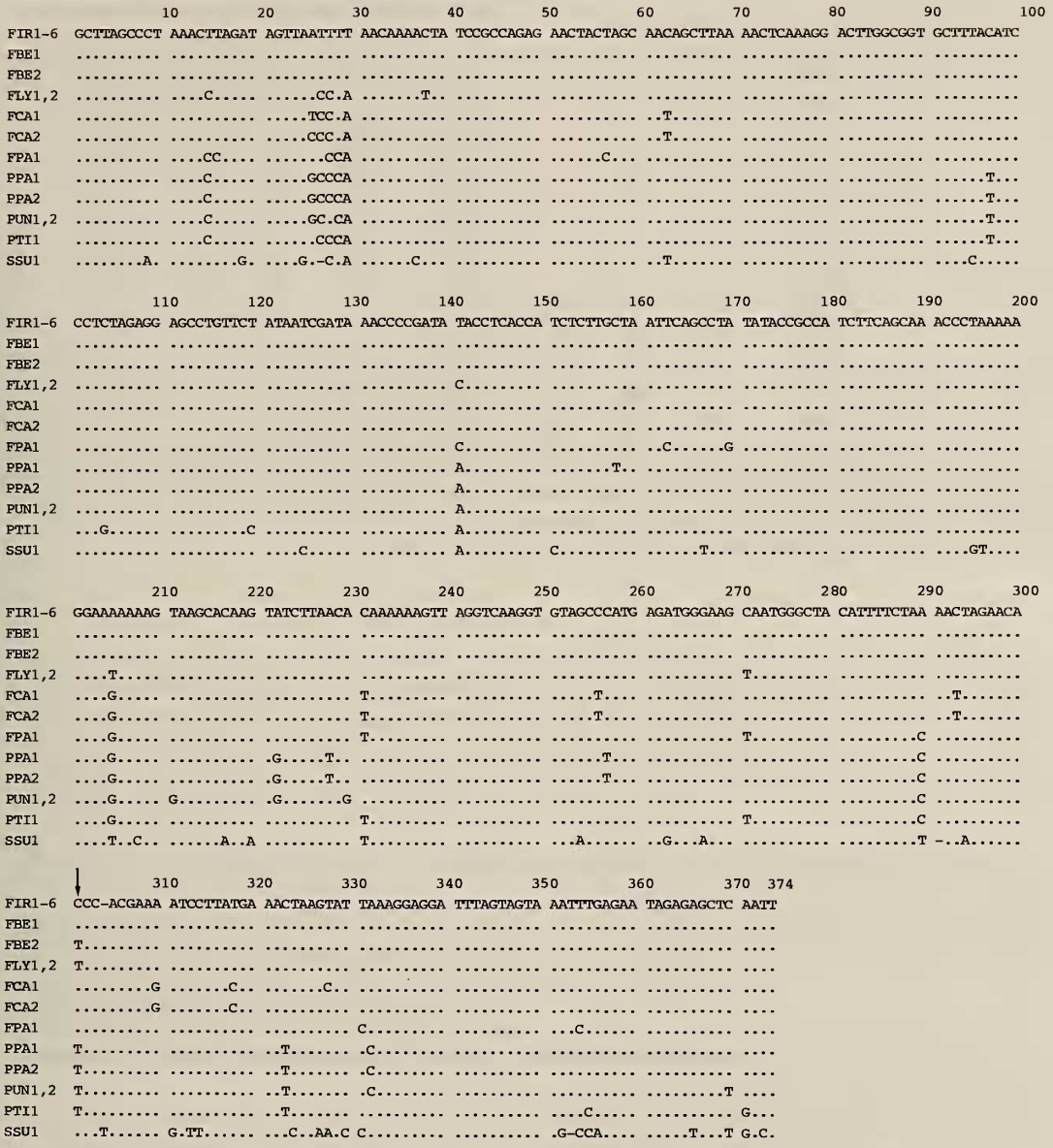

FIG. 1. Alignment of the 12S rRNA sequences from the Iriomote cat and other feline species. Species codes refer to those in Table 1. Dots indicate identities with nucleotides in the Iriomote cat seuqence (FIR1–6). Dashes denote gaps. The sequence of the Iriomote cat was identical with that of one leopard cat (FBE1). An arrow shows the substitution site, nt 301, between FIR1–6/FBE1 and another leopard cat (FBE2).

mtDNA sequences or the existence of mtDNA-like sequence(s) in the nuclear genome (data not shown). The cytochrome b region of FPA1 could not be amplified probably due to the mismatch of primer sequences, while the 12S rRNA region could be amplified from the same DNA sample.

No intraspecific variation was observed among the six Iriomote cats (FIR1–6) (Fig. 2). Two leopard cats (FBE1 and FBE2) shared identical sequences (Fig. 2). The sequence difference between the Iriomote cat and the leopard cat was 0.5% (2/402 bases) (Table 3). The two substitutions were transitional; G→A (FIR-FBE) at nt 108 and A→G at nt 354 (Fig. 2). Each substitution was synonymous and occurred at the third position in codon; one codon (CTG or CTA) including nt 108 encoded the proline and the other (ATA or ATG) including nt 354 encoded the methionine. Between the Iriomote cat and the other feline species excluding the leopard cat, the sequence differences were 10.4–13.2% (42–53/402 bases) (Table 3). The relationships between the transversional difference per site and the transitional difference per site were shown in Figure 3.

Phylogenetic trees

Phylogenetic trees of the 12S rRNA and the cytochrome b sequences were constructed using the neighbor-joining method, based on the numbers of nucleotide substitutions per site which were estimated by Kimura's two-parameter

Table 2. Percentage differences (above diagonal) and numbers of transitions/transversions (below diagonal) for mitochondrial 12S rRNA sequences (373 bases)

Code	FIR1-6*	FBE1	FBE2	FLY1,2*	FCA1	FCA2	FPA1	PPA1	PPA2	PUN1,2*	PTI1	SSU1**
FIR1-6*	—	0	0.3	2.4	3.2	3.0	4.0	4.6	4.3	4.3	4.6	10.8
FBE1	0/0	—	0.3	2.4	3.2	3.0	4.0	4.6	4.3	4.3	4.6	10.8
FBE2	1/0	1/0	—	2.1	3.5	3.2	4.3	4.3	4.0	4.0	4.3	11.1
FLY1,2*	7/2	7/2	6/2	—	3.8	3.5	3.5	3.8	3.5	4.0	3.2	11.1
FCA1	10/2	10/2	11/2	12/2	—	0.5	5.1	5.4	5.1	5.6	5.1	11.1
FCA2	9/2	9/2	10/2	11/2	2/0	—	4.8	5.1	4.8	5.4	4.8	11.1
FPA1	13/2	13/2	14/2	11/2	17/2	16/2	—	5.1	4.8	5.4	4.0	11.4
PPA1	12/5	12/5	11/5	9/5	15/5	14/5	16/3	—	0.3	1.9	3.2	13.0
PPA2	11/5	11/5	10/5	8/5	14/5	13/5	15/3	1/0	—	1.6	3.0	12.7
PUN1,2*	11/5	11/5	10/5	10/5	16/5	15/5	17/3	5/2	4/2	—	3.5	12.7
PTI1	13/4	13/4	12/4	8/4	15/4	14/4	13/2	9/3	8/3	10/3	—	11.4
SSU1**	27/13	27/13	28/13	30/11	28/13	28/13	31/11	36/12	35/12	35/12	31/11	—

* Six individuals of the Iriomote cat (FIR1-6), two of the lynx (FLY1 and FLY2), and two of the snow leopard (PUN1 and PUN2) showed no intraspecific sequence variation in each species.
** For comparison of SSU1 and feline species, three sites of gaps were eliminated.

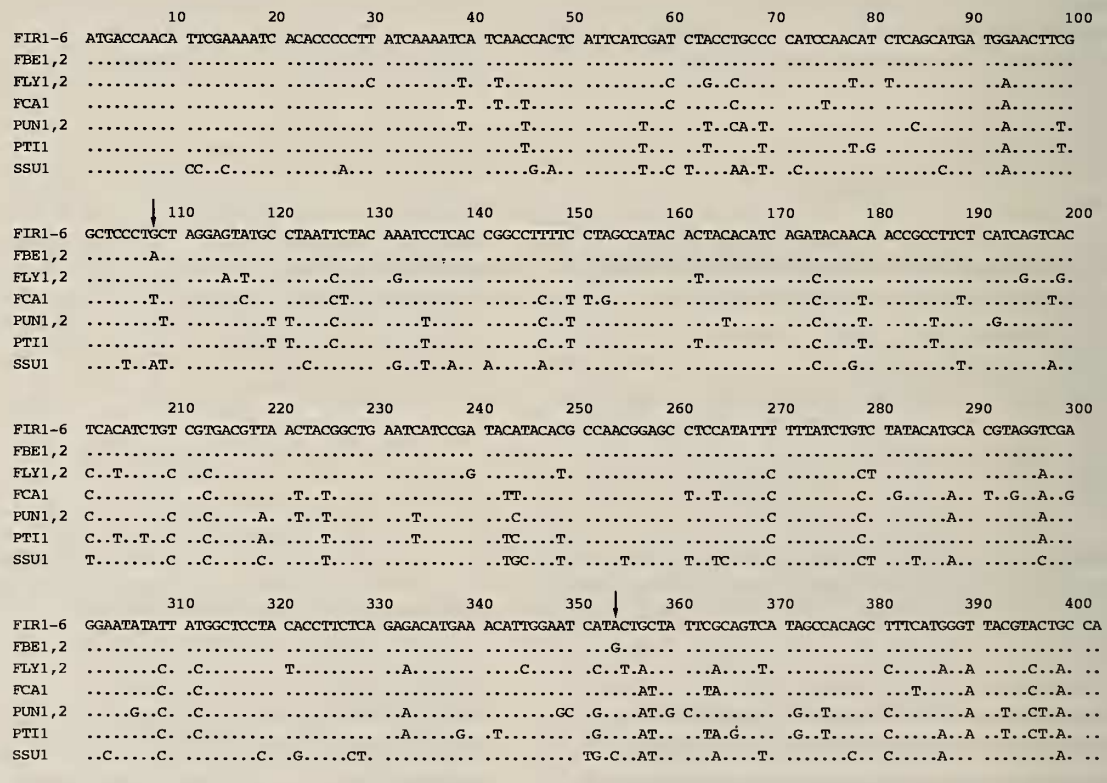

Fig. 2. Alignment of the cytochrome b sequences from the Iriomote cat and other feline species. Species codes refer to those in Table 1. Dots indicate identities with nucleotides in the Iriomote cat sequences (FIR1–6). Arrows show two substitution sites, nt 108 and nt 354, between the Iriomote cats (FIR1–6) and the leopard cats (FBE1 and FBE2).

method (Fig. 4). The Iriomote cats (FIR1–6) and the leopard cats (FBE1, 2) were clustered with 95% (the 12S rRNA tree) and 100% (the cytochrome b tree) bootstrap values. The species of the genus *Panthera* were clearly separated from the other felines with 76% (the 12S rRNA tree) and 100% (the cytochrome b tree) bootstrap values. Since the suricate (SSU1), which is included in the family Viverridae, was most distantly separated from the other species, SSU1 was used as an outgroup to set a root of the tree.

TABLE 3. Percentage differences (above diagonal) and numbers of transitions/transversions (below diagonal) for mitochondrial cytochrome b sequences (402 bases) of the Iriomote cat and other feline species

Code	FIR1–6*	FBE1,2*	FLY1,2*	FCA1	PUN1,2*	PTI1	SSU1
FIR1–6*	—	0.5	10.4	11.2	13.2	12.9	15.7
FBE1,2*	2/0	—	11.0	11.4	13.7	13.4	15.4
FLY1,2*	37/5	39/5	—	11.9	13.7	11.4	16.9
FCA1	39/6	40/6	45/3	—	12.2	12.4	16.9
PUN1,2*	46/7	48/7	49/6	44/5	—	6.7	18.2
PTI1	45/7	47/7	40/6	45/5	25/2	—	17.7
SSU1	46/17	45/17	46/22	45/23	49/24	47/24	—

* Six individuals of the Iriomote cat (FIR1–6), two of the leopard cat (FBE1 and FBE2), two of the lynx (FLY1 and FLY2), and two of the snow leopard (PUN1 and PUN2) showed no intraspecific sequence variation in each species.

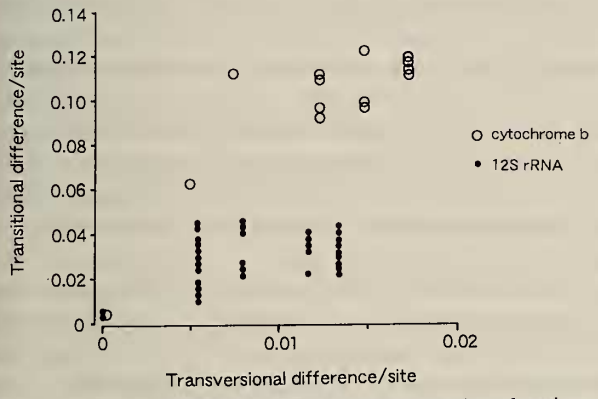

FIG. 3. The transversional difference per site was plotted against the transitional difference per site in pairwise comparisons at the 12S rRNA gene (closed circles) and the cytochrome b gene (open circles). The comparison with the suricate as an outgroup was not included.

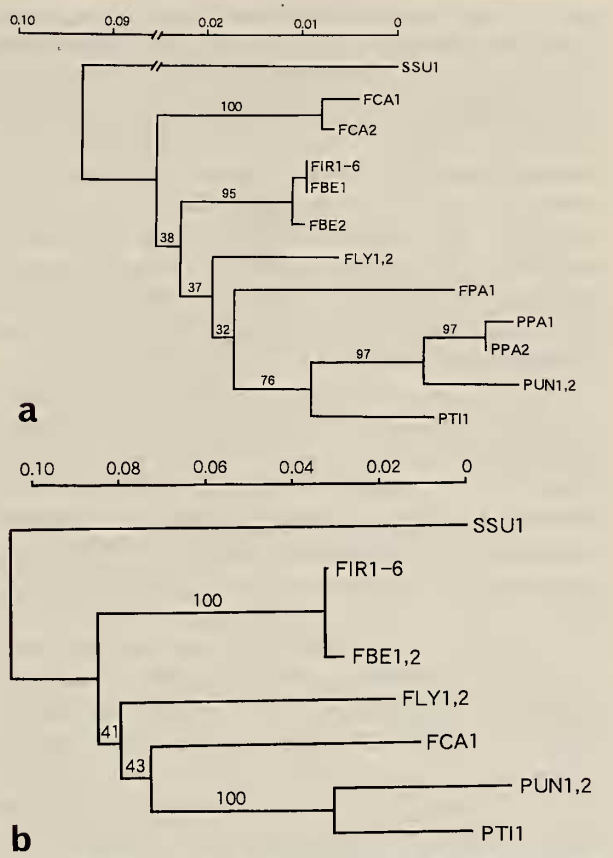

FIG. 4. Phylogenetic trees of the 12S rRNA (a) and the cytochrome b (b) sequences constructed by the neighbor-joining method. The numbers of nucleotide substitutions per site, indicated by the scale, were estimated using Kimura's two parameter method [18]. Bootstrap values above branches were derived from 500 replications.

DISCUSSION

MtDNA phylogeny of the feline species

From sequence comparisons, it was clarified that the saturation due to the multiple substitutions at the same site is being approached for transitions in the feline 12S rRNA gene (Fig. 3), similar to the previous reports of other animals [2, 4, 30]. In the cytochrome b gene, the multiple substitutions seem to have reached the saturation (Fig. 3). This result is in agreement with the previous observation [30] that the cytochrome b gene evolves faster than does the 12S rRNA gene in mammals.

Despite of the substitution rate difference between the two genes, the Iriomote cat (FIR1–6) and the leopard cat (FBE1, 2) were clustered and separated from the other feline species with high bootstrap values in both the 12S rRNA tree and the cytochrome b tree: 95% in the 12S rRNA tree and 100% in the cytochrome b tree (Fig. 4). This indicates that the Iriomote cat is closely related to the leopard cat with a high confidence. The *Panthera* species were clustered with high bootstrap values: 76% in the 12S rRNA tree and 100% in the cytochrome b tree (Fig. 4). Our results of the phylogenetic status of the *Panthera* species support the previous reports by the microcomplement fixation method [5] and the shorter sequences of the 12S rRNA gene [17]. The difference in topology between the 12S rRNA tree and the cytochrome b tree (Fig. 4) seems to reflect the substitution rate difference between the two genes and the absence of the ocelot (FPA) and the leopard (PPA) in the cytochrome b tree. Since the suricate (SSU1) from the family Viverridae was most distantly separated from the species of the family Felidae, this species was used to set a root of the tree.

MtDNA sequence similarity between the Iriomote cat and the leopard cat

The difference of the cytochrome b sequences was 0.5% (2/402 bases) between the Iriomote cat (FIR1–6) and the leopard cat (FBE1, 2) (Table 3, Fig. 2). The 12S rRNA sequences of six Iriomote cats (FIR1–6) were identical to that of one leopard cat (FBE1) and different by 0.3% (1/373 bases) from that of the other leopard cat (FBE2) (Table 2,

Fig. 1). The close relationship between the Iriomote cat and the leopard cat supports the previous report of Wurster-Hill *et al.* [36], in that the karyotypes of both felines were indistinguishable. However, it must be noticed that the karyological identity is not always in agreement with the molecular similarity. Wurster-Hill and Centerwall [35] reported that the species of the genus *Panthera* have identical karyotypes. By contrast with their karyological data, our molecular results indicated that the 12S rRNA sequence difference (1.6–3.5%, 6–13/373 bases) among the *Panthera* species is not always very low, as compared with the sequence difference among the other feline species (Table 2).

Between the two leopards (PPA1, 2) and between the two domestic cats (FCA1, 2), the intraspecific differences for the 12S rRNA sequences were 0.3% and 0.5%, respectively (Table 2). The sequence difference (0–0.3%) between the Iriomote cat (FIR1–6) and the leopard cat (FBE1, 2) is comparable to these intraspecific variations. Therefore, it is not reasonable for the two felines to be classified to distinct genera.

The leopard cat is widespread in Asia and has the geographic variation of the morphological characters [21]. Glass and Todd [8] reported on the presence or absence of the second premolar (P2) as a quasi-continuous variation in various leopard cat populations, while Imaizumi [12] described that the P2 is absent in the Iriomote cat. Moreover, Glass and Todd [8] considered that the Iriomote cat's key characters reported by Imaizumi [12] are polymorphic in the leopard cat, and suggested that the fixation of the P2 absence in the Iriomote cat population may show a result of the genetic drift in an island population [8]. Wozencraft [34] assigned the Iriomote cat to the synonym of the leopard cat based on the morphological characters (Table 4). Our results together with the previous taxonomic reports [8, 34] suggest that the geographic isolation has led the fixation of some unique morphological characters in the Iriomote cat population while both this feline and the leopard cat still retain very close genetic characters. Since the leopard cat is distributed in many islands of Asia, it is quite necessary to examine the genetic and morphological variations in each island population, and to compare them with the characters of the Iriomote cat population.

Divergence time of the Iriomote cat

In order to clarify the evolutionary origin of the Iriomote cat, it is important to estimate the divergence time of this feline from the leopard cat, which is a widespread species in Asia. Wayne *et al.* [33] estimated divergence time of various carnivore species based on the microcomplement fixation method. Their data showed that the divergence time between the domestic cat and the tiger is about 4.5 million years (Myr) ago. In our results, the sequence difference between the domestic cat (FCA1) and the tiger (PTI1) was 12.4% for the cytochrome b gene (Table 3). Based on these values, the substitution rate of the cytochrome b gene was calculated to be 1.38%/Myr. Between the Iriomote cat (FIR1–6) and the leopard cat (FBE1, 2), the sequence difference was 0.5% for the cytochrome b gene (Table 3). Using the calculated substitution rate and the sequence difference, the divergence time of the Iriomote cat and the leopard cat was estimated to be 0.18 Myr ago. On the other hand, since the cytochrome b gene was reported to diverge at a rate of at least 2.5%/Myr in mammals [13, 24], the Iriomote cat was estimated to have diverged from the leopard cat less than 0.20 Myr ago. Thus, the two divergence times estimated in different ways were in good agreement with each other.

Kimura *et al.* [19] with the geological data reported that the land bridge connecting the Ryukyu Arc and the Chinese continent existed intermittently from 0.24 to 0.02 Myr ago and that the land bridge subsided about 0.02 Myr ago. Our estimated divergence time of both felines is in agreement with the geological isolation date of the Ryukyu Arc. In fact, the leopard cats live in Taiwan, which is located about 200 km west of Iriomote Island [21]. It is still interesting that the Iriomote cat is distributed only on Iriomote Island among many islands of the Ryukyus. After isolation from the Chinese continent, the Ryukyu Islands may have undergone geological and environmental changes, and the ancestors of

TABLE 4. Nomenclatures of the Iriomote cat and the leopard cat, cited from previously proposed classifications

	Researcher (year)					
	Imaizumi (1967)	Ewer (1973)	Hemmer (1978)	Leyhausen (1979)	Nowak (1991)	Wozencraft (1993)
Iriomote cat						
Genus	*Mayailurus*	*Mayailurus*	*Prionailurus*	*Prionailurus*	*Felis*	Synonym of
Subgenus	—	—	*Mayailurus*	—	*Mayailurus*	the leopard cat
Species	*iriomotensis*	*iriomotensis*	*iriomotensis*	*iriomotensis*	*iriomotensis*	
Leopard cat						
Genus		*Prionailurus*	*Prionailurus*	*Prionailurus*	*Felis*	*Prionailurus*
Subgenus		—	*Prionailurus*	—	*Prionailurus*	—
Species		*bengalensis*	*bengalensis*	*bengalensis*	*bengalensis*	*bengalensis*
Reference	[12]	[6]	[10]	[22]	[25]	[34]

the Iriomote cats may have been able to survive only on Iriomote Island. Further paleontological studies will elucidate their past distribution on the islands.

Controversy on nomenclatures in the Felidae classification

The Iriomote cat was reported to have primitive morphological characters and was named *Mayailurus iriomotensis* as a new genus and a new species [12]. The genus name of the Iriomote cat was, however, different among several versions of the Felidae classifications presented so far. In Table 4, the nomenclatures of the Iriomte cat and the leopard cat were extracted from the previous classifications. Our results based on mtDNA sequence analysis support the classifications in that the Iriomote cat is included in the same genus as the leopard cat. As shown in Table 4, Hemmer [10], Leyhausen [22], and Nowak [25] all classified the two felines to one genus, but there is some disagreement between their classifications on the other feline species, especially the *Panthera* species. Hemmer [10] assigned the snow leopard to *Uncia uncia*, while the leopard and the tiger were named *Panthera pardus* and *P. tigris*, respectively. In Leyhausen's classification [22], the snow leopard was assigned to *Uncia uncia*, while the leopard was *Panthera pardus* and the tiger was *Neofelis tigris*. Thus, Hemmer [10] and Leyhausen [22] divided these three big felines into two or three genera. By contrast, Nowak [25] included all of these big felines in one genus *Panthera*. In our results, the *Panthera* species, which have identical karyotypes [35], were clustered with a high confidence (Fig. 4). Considering the classification throughout the feline species, therefore, Nowak's classification [25] was adopted in this report, although he classified the Iriomote cat and the leopard cat into the different subgenera, *Mayailurus* and *Prionailurus*, respectively.

This is the first report on the molecular phylogeny of the Iriomote cat, inferred from mtDNA sequences. The phylogenetic understanding is the basis and the first step to know the genetic diversity of the Iriomote cat population for the purpose of conservation and management.

SEQUENCE AVAILABILITY

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the following accession numbers: for the 12S rRNA sequences, D28888 (FIR), D28889 (FBE1), D28890 (FBE2), D28891 (FLY), D28892 (FCA1), D28893 (FCA2), D28894 (FPA), D28895 (PPA1), D28896 (PPA2), D28897 (PUN), D28898 (PTI), D28899 (SSU): for the cytochrome b sequences, D28900 (FIR), D28901 (FBE), D28902 (FLY), D28903 (FCA), D28904 (PUN), D28905 (PTI), D28906 (SSU).

ACKNOWLEDGMENTS

We thank Dr. M. Izawa, Department of Biology, University of the Ryukyus, for supplying specimens and invaluable advice of the Iriomote cat. We are grateful to R. Kuwae and T. Kinjo, Okinawa Children Land Zoo, and K. Murata, Kobe Oji Zoo, for contribution of specimens. We are also indebted to Dr. M. Yoneda, Japan Wildlife Research Center, for valuable advice and Dr. M. Kimura, University of the Ryukyus, for geological suggestion. All specimens of the Iriomote cats were examined under the permission of the Envionmental Agency and the Agency for Cultural Affairs of Japan. This work was supported in part by a research grant from the Sumitomo Foundation.

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