Phylogenetic relationship between the Iriomote cat and the leopard cat, *Felis bengalensis*, based on the ribosomal DNA

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(Received 7 April 1994)

ABSTRACT

We analyzed the restriction fragment length polymorphisms in the spacer regions of ribosomal DNA (rDNA), using twelve restriction enzymes, to examine whether the Iriomote cat is related to the leopard cat (*Felis bengalensis*). A restriction map for each taxon was constructed and the major taxon-specific types of repeating unit (repetypes) were characterized on the basis of the arrangements of restriction sites. The Iriomote cat and the leopard cat share a common repetype but this repetype is different from that of the domestic cat (*F. catus*) with an estimated sequence divergence of 1.5% and from that of the ocelot (*F. pardalis*) with an estimated sequence divergence of 2.5%. These results indicate that, phylogenetically, the Iriomote cat is closely related to the leopard cat and that the ancestral population moved from the continent to Iriomote Island quite recently. The rDNA arrays of the leopard cat exhibit considerable intragenomic size-variation, which is thought to have emerged as a result of differences in numbers of repeated DNA segments, whereas the extent of such size-variation is much lower in the rDNA of the Iriomote cat. It appears that, even though migration of the Iriomote cat occurred relatively recently, the population has diverged to some extent from its continental counterpart, perhaps via fixation of preexistent intraspecific variations rather than by generation of new variations.

1. INTRODUCTION

In 1965, the Iriomote cat was recognized as an indigenous inhabitant of Iriomote Island, which is located in the southern-most part of the Ryukyu Islands, near

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Taiwan. Imaizumi (1967) investigated the morphological characteristics of specimens and reported a new species, *Mayailurus iriomotensis*, concluding that the species is distinct from other species of wild cats, such as the leopard cat, *Prionailurus* (= *Felis*) *bengalensis*, which is distributed widely in eastern parts of the Asian continent, including East Siberia, Taiwan, and Borneo, and Tsushima Islands, Japan. However, some taxonomists have claimed that the classification is incorrect; they have defined the Iriomote cat as a species that is closely related to the leopard cat and named it *Felis iriomotensis* (Corbet and Hill, 1991). Wozencraft (1993) considered the leopard cat and the Iriomote cat to be synonymous since most of Imaizumi's key characteristics are also included in the polymorphic characteristics observed in populations of the leopard cat (Glass and Todd, 1977). In addition, cytogenetic studies have also revealed a closer relationship: the Iriomote cat and the leopard cat have similar karyotypic morphology (Tsuchiya, 1976, 1979) and identical patterns of G-banding (Wurster-Hill et al., 1987; Modi and O'Brien, 1988).

The phylogenetic relationship between the two taxa has not been examined in molecular terms. In the past decade, qualitative phylogenetic analyses of related species have been performed at the DNA level, based on variations in nuclear genomes. In particular, it has been shown that restriction enzyme fragment length polymorphism (RFLP) of the spacer regions of ribosomal DNA (rDNA) provides a good tool for estimations of phylogenetic relationships between related species (Arnheim et al., 1980; Wilson et al., 1984; Suzuki et al., 1986, 1987, 1990, 1994a,b; Allard and Honeycutt, 1991; Wall et al., 1992; Hosoda et al., 1993; Lan et al., 1993; Amano et al., 1994).

The rDNA locus exists as a multigene family which consists of several hundred copies. These copies are usually separated into tandemly repeated clusters located at several different sites on chromosomes. Each repeating unit of rDNA is composed of three genes for rRNA, namely, genes for 18S, 5.8S, and 28S RNA, which are separated from each other by spacers. The spacers are known to evolve rapidly (Arnheim, 1983), and RFLPs are generated predominantly by base changes at restriction sites and by the deletion, insertion, or duplication of DNA segments. It is considered that homologous regions on different rDNA arrays evolve concertedly (Arnheim et al., 1980; Ohta, 1980; Dover, 1982) but different regions of the same rDNA array evolve independently (Suzuki et al., 1994a). Thus, a survey of RFLPs of rDNA from small numbers of samples can provide reliable information about the extent of phylogenetic divergence between a given pair of taxa.

We constructed restriction maps of an approximately 40-kb portion of the repeating units of rDNA, encompassing the rRNA-coding region, the internal spacer regions and the flanking spacer regions, using three different probes. We found that RFLPs of the rDNA of the Iriomote cat were very similar to those of the leopard cat.

2. MATERIALS AND METHODS

Samples of DNA

Specimens of the Iriomote cat were examined with the permission of the Environmental Agency and the Agency for Cultural Affairs of Japan. A total of four Iriomote cats, each of which had died in a traffic accident, was used. One leopard cat, which had been collected in the Primorie region (East Siberia), Russia, was used. Nuclear DNA was prepared from livers as described by Maniatis et al. (1982). Samples of DNA from the domestic cat and the ocelot, stored at the Miyazaki Medical College and Chiba Zoological Park, respectively, were used as reference materials for the phylogenetic study.

Construction of restriction maps

Southern blot analysis was carried out as described by Suzuki et al. (1990). To construct the restriction maps for the various types of rDNA repeating unit (repetypes), the genomic DNAs of the specimens were subjected to digestion with 12 restriction enzymes, namely, *Aat*I, *Bam*HI, *Bcl*I, *Bgl*II, *Dra*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, and *Xba*I. The digested DNAs, after electrophoresis and transfer to nylon filters, were allowed to hybridize sequentially with three ³²P-labeled rDNA probes, designated 18SB, 28S, and INT, respectively (see Fig. 2). These probes were prepared from clones of mouse rDNA as described by Kominami et al. (1981, 1982). Autoradiography and the quantitative analysis of the radioactivity of hybridized bands of DNA were performed with an image analyzer (BAS2000; Fuji Film Co., Tokyo). From the patterns of Southern blotting, restriction maps for the coding and internal spacer regions were constructed. Then restriction sites were mapped on the external spacers that flank the genes for 18S or 28S RNA. Each of the restriction maps that we constructed is considered to represent a major type of repeating unit (reptype) in each taxon.

Estimation of sequence divergence

We compared the arrangements of restriction sites between pairs of rDNA repetypes and counted the common and different sites. Polymorphic sites that exhibited intracellular heterogeneity were treated as half common and half different for convenience. Since restriction sites in the coding region and the *Dra*I and *Kpn*I sites in the internal spacers are basically conserved in mammalian rDNAs, these sites were excluded from the comparison. Employing a method developed by Gotoh et al. (1979) in which backward mutations and parallel mutations are taken into account (Jukes and Cantor, 1969), we calculated the sequence divergence among the various repetypes.

3. RESULTS

Southern blotting was performed (see Fig. 1, for examples) and restriction maps were constructed (Fig. 2) for the four taxa of cats listed in Table 1. Sequence divergence was estimated on the basis of the arrangement of restriction sites (Table 1). Hybridization patterns for the Iriomote cat were very similar to those for the leopard cat from East Siberia (Fig. 1). Among a total of 29 restriction sites on the spacers examined, not one change was observed. Thus, these two taxa are considered to share the same repetype. By contrast, when we compared the repetype of the leopard cat with that of the domestic cat, four out of the 29 restriction sites were completely different from the respective counterparts. Digestion with *Dra*I of the DNA from the domestic cat yielded two bands that hybridized with the 18SB probe; one of the bands was common to the leopard cat and the other was specific to the domestic cat (Fig. 2b). Consequently, the repetype of the leopard cat was estimated to be different from that of the domestic cat with a sequence divergence of 1.5% (the numbers of common and different sites were counted as 24.5 and 4.5, respectively; Table 1). We also analyzed the rDNA of the ocelot, which is a member of the genus *Felis* (Corbet and Hill, 1991) and was used as a reference in this study. The sequence divergence between the repetypes of the leopard cat and the ocelot was estimated to be 2.5% (Table 1).

Fig. 1. Examples of Southern blot patterns obtained with the 0.7-kb 28S probe (a) and the 0.9-kb 18SB probe (b). See Fig. 2 for the probes. The Iriomote cat (lane 1), the leopard cat (lane 2), the domestic cat (lane 3) and the ocelot (lane 4) are compared. The restriction enzymes used are indicated above each blot. Sizes (kb) of bands are indicated with arrowheads.

Fig. 2. Restriction maps of the major rDNA repetypes of the four taxa of cats. The open boxes indicate the genes that encode 18S, 5.8S and 28S rRNA, respectively. (a) The diagram shows the conserved restriction sites in the coding and the internal spacer regions. Positions of probes are shown with double-headed arrows. (b) Restriction maps of the spacer region of the rDNA of the leopard cat (1), the domestic cat (2), and the ocelot (3). The repetype of the Iriomote cat is the same as that of the leopard cat. With respect to the restriction sites on the flanking spacers, only those nearest to the distal end of the genes for 18S (upper part) and 28S (lower part) RNA are shown. Asterisks indicate polymorphic sites within an individual. In some individuals, size variations were detected and such regions are marked with black triangles. A, *Aat*I; B, *Bam*HI; D, *Dra*I; E, *Eco*RI; G, *Bgl*II; H, *Hind*III; K, *Kpn*I; L, *Bcl*I; P, *Pst*I; S, *Sac*I; V, *Pvu*II; X, *Xba*I.

Table 1. Sequence divergence among the major repetypes of rDNA from four taxa of wild cats (upper right), based on numbers of common and different restriction sites (lower left)

Taxon*	Sequence divergence (%)**			
	1	2	3	4
1. Iriomote cat (<i>F. iriomotensis</i>)	-	0	1.5	2.5
2. Leopard cat (<i>F. bengalensis</i>)	29/0	-	1.5	2.5
3. Domestic cat (<i>F. catus</i>)	24.5/4.5	24.5/4.5	-	1.2
4. Ocelot (<i>F. pardalis</i>)	22/7	22/7	24.5/3.5	-

* According to Corbet and Hill (1991).

** Total number of sites (common/different) for the spacer upstream from the gene for 18S rRNA, the internal spacer regions and the spacer region downstream from the gene for 28S rRNA.

The length polymorphisms within the genome, which were due to variations in the external spacer just downstream of the 3' end of the gene for 28S rRNA (marked with black triangles in Fig. 2b), were remarkable in the genomes of the leopard cat and the domestic cat. For example, *Bam*HI bands hybridizing with the 28S probe appeared to be polymorphic within the single leopard cat, whereas the comparable fragments from the Iriomote cat exhibited less polymorphism within the respective genomes of the four individuals examined (Fig. 3). These results suggest that the Iriomote cat differs to some extent from the leopard cat, possibly as a result of fixation of the polymorphism within the genome and within the population during the course of populational isolation after the cats migrated to Iriomote Island.

Fig. 3. Southern blotting patterns, after digestion of DNAs with *Bam*HI, obtained with the 28S probe. The Iriomote cat (lanes 1-4), leopard cat (lane 5), and domestic cat (lane 6) are compared. Sizes (kb) of bands are indicated with arrowheads. Quantification of the radioactivity associated with each band was performed with an image analyzer. Relative radioactivities (%) of the hybridized fragments in each lane are shown in parentheses.

4. DISCUSSION

In this study we examined RFLPs in the spacer regions of the rDNA from four taxa of cats, namely the Iriomote cat, the leopard cat, the domestic cat, and the ocelot, where some taxonomical confusion still exists. We found that the Iriomote cat, which was classified as a distinct species soon after its discovery (Imaizumi, 1967), retains the same arrangement of restriction sites as the leopard cat. Thus, it appears that, phylogenetically, the Iriomote cat is closely related to the leopard cat. By contrast, the domestic cat differs from these two cats with a sequence divergence of 1.5%. The close phylogenetic relationship between the Iriomote cat and the leopard cat reflects the close relationship that was proposed on the basis of morphological (Glass and Todd, 1977; Wozencraft, 1993; Corbet and Hill, 1991) and karyological (Wurster-Hill et al., 1987; Tsuchiya, 1976, 1979; Modi and O'Brien, 1988) studies. Our results are also consistent with results obtained

from a study of mitochondrial DNA (Masuda et al., 1993), in which sequences from the Iriomote cat were found to be very similar to those from the leopard cat.

Previously we analyzed a variety of species of Carnivora and estimated the evolutionary rate of divergence to be 1.86% per million years (Myr) from the value of the sequence divergence of 8.6% between the fox and the raccoon dog (Hosoda et al., 1993), which are considered to have diverged about 5 Myr ago (Wayne et al., 1991). We also examined the sequence divergence in higher primates and found that the sequence divergence between the human and the chimpanzee was 2.5% (Suzuki et al., 1994b). From these data, the rate of divergence of rDNA spacers can be estimated to be 0.5%–2% per Myr. When 29 restriction sites are examined, one restriction site difference corresponds to a sequence divergence of 0.33% and to 0.17–0.66 Myr since the time of divergence. Therefore, we can postulate that the Iriomote cat migrated from the continent to Iriomote Island quite recently, perhaps during the middle or late Pleistocene.

The geographic history of the Ryukyu Islands is still ambiguous. Unlike the dispersal of house mice or rats, whose migration depends largely on human actions, dispersal of wild cats is thought to be rather independent of such influences. Thus, the results of our analysis of the genetic differences between the isolated population on Iriomote Island and those on the continent may contribute to an estimation of the time of the geographic isolation of the island. Our results strongly suggest that the island was connected to the continent by a land bridge at least once within the past half Myr.

As predicted from the fact that the Iriomote wild pig exhibits low genetic variability (Kurosawa et al., 1989), the Iriomote cat was found to exhibit low intragenomic size-variation in its rDNA spacers (Fig. 3). Size variations are often associated with local, highly variable regions that contain repetitive DNA and have been well documented in rDNAs of the human (Arnheim et al., 1980; Krystal et al., 1981) and the mouse (Arnheim et al., 1982; Kuehn and Arnheim, 1983). We showed previously that the extent of intragenomic heterogeneity of rDNA is largely dependent on the size of the population to which individuals belong (Suzuki et al., 1994a). In the case of the Japanese field mouse, *Apodemus speciosus*, populations on small islands are inclined to have fixed types of polymorphic restriction fragment and such fixation is thought to have been accomplished over the course of $1-2 \times 10^5$ years since the isolation of the small islands from the mainland of Japan (Suzuki et al., 1994a). We can calculate the intragenomic heterogeneity from the following equation:

$$h = 1 - \sum x_i^2$$

(where x_i is the relative frequency of the i th fragment).

In the case of the Iriomote cat, the extent of such heterogeneity is low ($h = 0$). In the case of the individuals examined here, the values for the leopard cat ($h = 0.63$) and the domestic cat ($h = 0.41$) were rather high. We assume that the

reduction in heterogeneity may be due to the isolation of the population of Iriomote cats for a considerable length of time and with only a limited number of individuals. The size of the contemporary Iriomote cat population has been estimated to be 100 individuals (Izawa et al., 1990). With respect to the reduction in polymorphism in the Iriomote cat, from their comparative study of morphological characteristics of the leopard cat, Glass and Todd (1977) concluded that the Iriomote cat is an island form of the leopard cat and lacks polymorphism within the island population. They emphasized that the fixation of morphological characteristics in the Iriomote cat can be explained as a result of genetic drift.

The population on Iriomote Island seems to have diverged to a specific form from the continental populations via loss of heterogeneity during the course of its isolation in the small island. Iriomote-specific combinations of morphological characteristics (Imaizumi, 1967; Grove, 1982) and ecological behavior (e.g. Yasuma, 1981) have been reported and such features may have accumulated during the period of isolation from the mainland.

The authors are very grateful to Mr. N. Sakaguchi, Drs. M. Izawa and V. G. Yudin for providing samples of livers; Drs. K. Moriwaki, R. Masuda, M. Izawa and A. P. Kryukov for valuable comments on this manuscript; Drs. R. Kominami and M. Muramatsu for providing the mouse rDNA clones; and Miss R. Nakayama for her technical assistance. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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