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Genetic Variation of the Mitochondrial DNA Cytochrome *b* Region in Japanese Native Dog Breeds (*Canis familiaris*)

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ABSTRACT—Partial sequences (454 bases) of the mitochondrial DNA (mtDNA) cytochrome *b* gene (*cyt b*) were determined for 94 dogs including 73 Japanese native animals. Thirteen nucleotide positions of this region showed nucleotide substitutions, which were all transitions. Three of 13 nucleotide substitutions were nonsynonymous. A total of 14 *cyt b* haplotypes were found, but the Japanese native dog breeds could not be differentiated as distinct clusters in phylogenetic trees. These results support the previous view that genetic variations observed among Japanese native dog breeds could have resulted from interbreeding and/or intrabreeding.

INTRODUCTION

Most Japanese native dog breeds were raised as hunting dogs to catch wild boars, deer and bears in several mountainous districts for a long time. Based on their original localities and physical characters, the following six breeds have been preserved by their respective kennel clubs and have been specified as natural monuments of Japan: Hokkaido dog of medium size from Hokkaido Island; Akita dog of large size from Akita Prefecture; Kai dog of medium size and tiger brindle coat color from Yamanashi Prefecture; Shiba dog of small size from Honshu island; Kishu dog of medium size from Kii Peninsula and Shikoku dog of medium size from Shikoku Island. Also, the local dog breeds of Iki Island (Iki dog) and Okinawa Islands (Ryukyu dog) have been preserved in their regions. Tanabe *et al*. (1991) investigated 25 blood protein loci of about 3,000 specimens of 40 breeds or local dog populations. They reported that six Japanese breeds are genetically different from foreign breeds, and that the Hokkaido and Ryukyu breeds are more aboriginal than the other five Japanese breeds that have been maintained for a long time on Honshu main island.

Mitochondrial DNA (mtDNA) polymorphism has been widely used to study the relationships between closely related species and the genetic structure within populations (Harpending, 1994; Macmillan and Bermingham, 1996). Our previous study (Okumura *et al*., 1996) of the whole mtDNA noncoding region of about 94 dogs showed four phylogenetic clusters whose lineages did not directly agree with distinct dog breeds. To further examine the molecular lineages between the dog breeds, in this study, we determined partial DNA sequences of the mitochondrial cytochrome *b* gene (*cyt b*), because the protein-coding regions, such as *cyt b*, has a slower evolutionary rate than the mtDNA noncoding region.

MATERIALS AND METHODS

Ninety-four dog specimens comprising 73 Japanese native dogs and 21 non-Japanese dogs (Okumura *et al*., 1996) were used in this study. Total DNA was isolated from peripheral blood leukocytes using a nuclear lysis solution containing proteinase K (1 mg/ml) (Ishiguro *et al*., 1994). The *cyt b* gene was amplified by PCR using two primers, mitL68: 5'-CTTACTACACAATCAAGGATAT(15199) and mitH67: 5'-TTACTCTCCATTTTTGGTTTAC(15688), using the GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA). The number in parentheses is the 3'-end position of the corresponding sequence of bovine mtDNA (Anderson *et al*., 1982), and L and H are the light and heavy strands, respectively. Amplification of the *cyt b* region was performed with the same condition as described by us (Okumura *et al*., 1996). The PCR product was electrophoresed on 1% low melting agarose gels (NuSieve: Takara Shuzo, Kyoto, Japan), purified with phenol and chloroform, and precipitated by ethanol. The purified PCR products were directly sequenced by a 373S DNA Sequencer with a Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA). The DNA sequence data were analyzed using GENETYX-MAC software (Software Development CO., LTD., Tokyo) for multiple sequence alignment.

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RESULTS AND DISCUSSION

Figure 1a shows a representative 454 base *cyt b* sequence from one Shiba dog, and Fig. 1b shows nucleotide variations among 94 dogs of all breeds. A total of 14 haplotypes were found and designated by a to n (Fig. 1b, Table 1). The nucleotide substitutions at positions 218, 357 and 384 were nonsynonymous, whereas the others were synonymous. Haplotype a was predominantly observed (38%, 36/94 dogs), while the frequencies of haplotypes c, j and l were approximately 13%, 14% and 16%, respectively. The mtDNA lineages did not agree with the breeding lineages from the distribution of the *cyt b* haplotype (Table 1).

A phylogenetic analysis was made using the PHYLIP program package, ver 3.572 (Felsenstein, 1995). The number of nucleotide substitutions per nucleotide site between the sequences was calculated by the two-parameter method (Kimura, 1980). A dendrogram was constructed using the

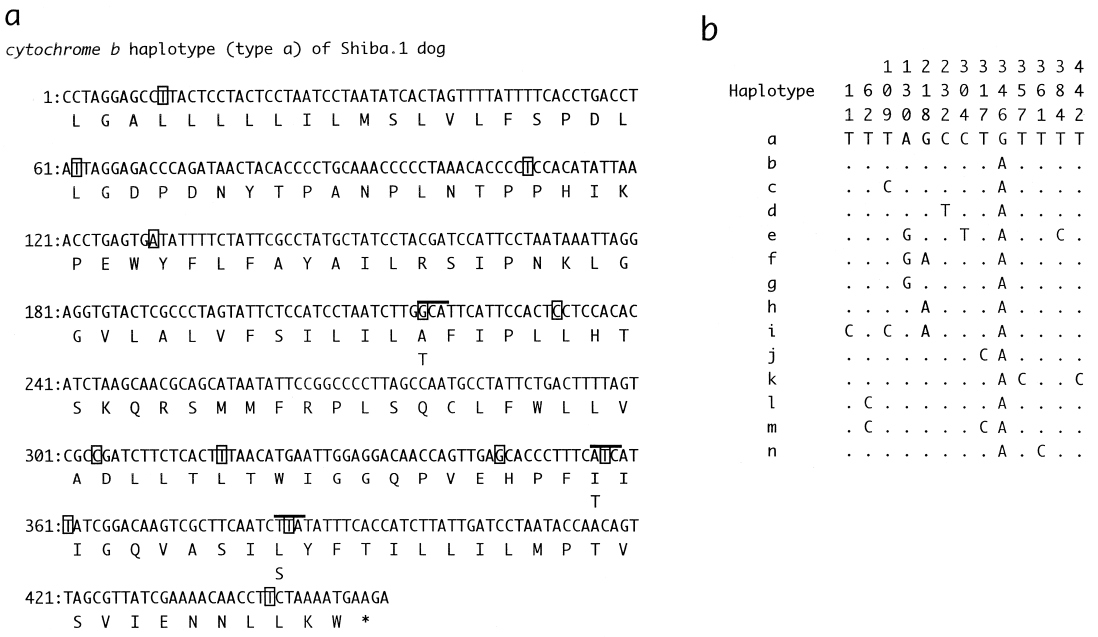

Fig. 1. (**a**) The 454 base cytochrome *b* sequence of Shiba.1 dog. The variable nucleotide positions are shown by open boxes. Bars above the sequences indicate codons showing nonsynonymous mutations. The deduced amino acid sequences are given in one-letter code. (**b**) Nucleotide substitutions of the cytochrome *b* region among 94 dogs. Dots indicate nucleotide sequences matching those of the haplotype "a", and the nucleotide position numbers on the top of the figure correspond to those of the above sequence. The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence data bases with accession numbers D87692 to D87705.

Table 1. The relationships between dog breeds and *cyt b* haplotypes

Haplotypes	No. of dog breeds									Total	Noncoding region cluster*
	Shiba	Akita	Hokkaido	Ryukyu	Kishu	Iki	Shikoku	Kai	Non-Japanese		
a	16	2	2	4	–	3	–	1	8	36	C1
b	–	–	–	1	–	3	–	–	2	6	C1,C4**
c	1	1	3	–	2	–	1	–	4	12	C1
d	–	–	–	1	–	–	–	–	–	1	C1
e	–	2	–	–	–	–	–	–	–	2	C1
f	–	–	–	–	1	–	–	–	–	1	C1
g	–	–	–	2	–	–	–	–	–	2	C1
h	–	–	–	–	–	–	–	–	1	1	C1
i	1	–	–	–	–	–	–	–	–	1	C1
j	9	–	–	1	–	–	1	–	2	13	C2
k	–	–	–	–	1	–	–	–	1	2	C3
l	–	4	3	3	1	2	–	–	2	15	C4
m	–	–	–	–	–	–	–	–	1	1	C4
n	1	–	–	–	–	–	–	–	–	1	C4
Total	28	9	8	12	5	8	2	1	21	94	

* Noncoding region clusters were cited from our previous study of these 94 modern dogs using the UPGMA method (Okumura *et al.*, 1996).
** One Ryukyu dog with the "b" was included in the C4 lineage of our previous study (Okumura *et al.*, 1996).

neighbor-joining method (Saitou and Nei, 1987). The bootstrap method was used to determine the confidence intervals of the phylogenies from 1,000 replications.

Figure 2 shows a neighbor-joining tree of the 14 *cyt b* haplotypes. The diversity of *cyt b* haplotypes could not be classified into clusters (Fig. 2). This branching pattern was different from that of the noncoding region lineage which had four clusters (Okumura *et al.*, 1996). The result may have been caused by the shorter sequences and lower divergence in the *cyt b* genes compared with approximately 980 base noncoding region of our previous study (Okumura *et al.*, 1996). The sequence analysis of the noncoding region is likely more useful than that of the *cyt b* gene to understand the interbreed genetic variation.

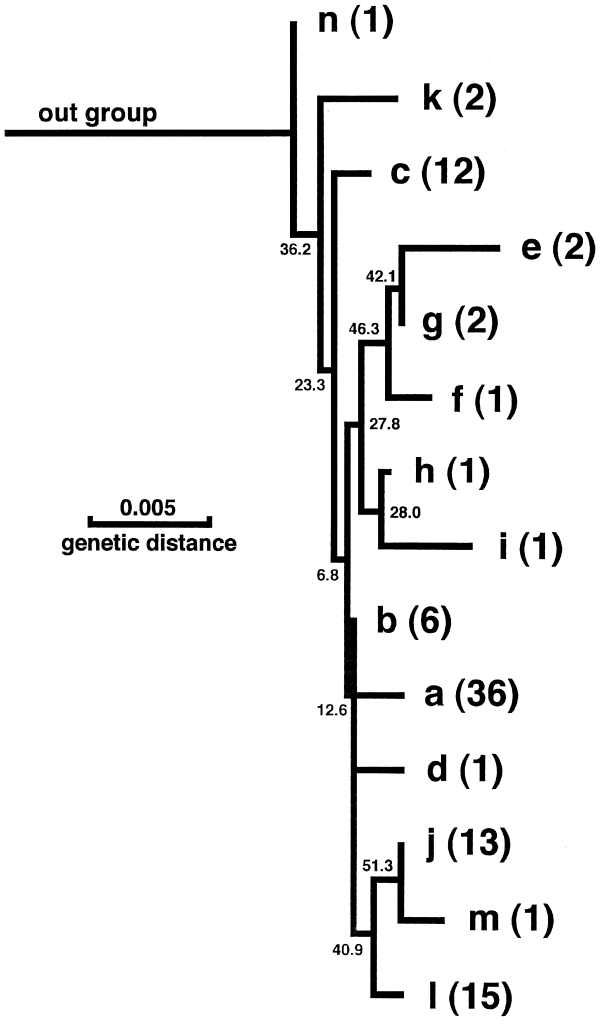

Fig. 2. A neighbor-joining tree reconstructed from indices of the nucleotide substitution per site calculated by the Kimura's two-parameter method (1980) among 94 sequences of canine mtDNA partial cytochrome *b* regions. The homologous region of the Japanese red fox (*Vulpes vulpes japonica*) was used as an outgroup. The designation of each haplotype is the same as shown in Table 1. The bootstrap probabilities (%) derived from 1,000 resamplings are shown above internal branches.

The nucleotide diversities in the Japanese native breeds, estimated by the method of Nei and Li (1971), varied from 0.106% (within Iki dogs) to 0.287% (within Kishu dogs), while the net nucleotide differences varied from 0.119% (between Ryukyu and Iki dogs) to 0.327% (between Akita and Iki dogs). That the nucleotide diversities are similar to the net difference is reasonable, because members of each dog breed sporadically belonged to the mtDNA lineages. The mtDNA lineages did not directly agree with distinct dog breeds. These results indicate interbreeding in the Japanese native dog breeds and support our previous study of the mtDNA noncoding region (Okumura *et al.*, 1996).

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