NOTE *Clinical Pathology*

Lineage Classification of Canine Inheritable Disorders Using Mitochondrial DNA Haplotypes

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ABSTRACT. To estimate the maternal effects of dog breeds using mitochondrial DNA(mtDNA) haplotypes in the dogs with several clinical disorders, 600 base pairs of mtDNA D-loop region were amplified from 365 dogs and were determined for mtDNA sequences. The diversity of the 600-bp sequences was classified into 64 haplotypes, including 46 newly discovered haplotypes, and the haplotypes were grouped into four clusters I to IV. Lineage analysis using the mtDNA haplotype indicated that each dog breed genetically comprises one or a few mtDNA haplotypes. When the relationship between genetic background and occurrences of clinical diseases was estimated, canine lineage analysis using mtDNA haplotype revealed that the disorders distributed in the dominant mtDNA haplotypes of each dog breed, but no disorder closely associated with mtDNA haplotypes was detected.

KEY WORDS: D-loop, haplotype lineage analysis, mitochondrial DNA.

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The domestic dog (*Canis familiaris*) is thought to have been domesticated from only one species, the wolf (*Canis lupus*) [11, 12]. More than 400 dog breeds have been established during the history of domestication with or without artificial selections [2]. To examine the phylogenetic relationships and to measure the variability among dog populations, mtDNA polymorphisms have been used widely, because mtDNA has strict maternal inheritance and lack of genetic recombination [1, 6]. A phylogenetic analysis of the mtDNA non-coding region (D-loop region) showed that dogs are classified into four distinct clusters and each dog breed has many haplotypes in the distinct clusters [8, 11]. However, distinct mtDNA haplotypes were sometimes in the different dog breeds. Specific dog breeds are inclined to have several diseases, but, the mode of inheritance and molecular basis of canine inherited or familial diseases remain unsolved. It is of interest to examine how the inheritable disorders are associated with certain dog breeds from the restricted area. In this study, the canine mtDNA haplotype of the dogs with several disorders were examined, and then the relationship between genetic background and occurrence of clinical diseases was examinied to evaluate the maternal effects of dog breeds.

Whole blood samples were collected from 365 patient dogs that received several clinical treatments in the Veterinary Teaching Hospital of Obihiro University or seven veterinary private clinics in Obihiro. Individual information of the patient dogs (breed, age, sex, and clinical diagnosis) were collected in this study. Genomic DNA was isolated from peripheral blood leucocytes [5]. Approximately 0.05–0.1 μg of the genomic DNA was used to amplify the mtDNA D-loop regions by PCR. Two primers, mit3 (ATATACTGGTCTTGTAAACC) and mit123 (AAACTATATGTCCTGAAACC), were used to amplify 600 base pairs (600-bp) of the D-loop region. After amplification, the DNA fragments were purified and sequenced directly by the dideoxy chain termination method using a 373A DNA Sequencer with a Taq DyeDeoxy Terminator Cycle Sequence Kit (Applied Biosystems Faster city, CA). Two additional primers, mit132 (5'-TAAGGGCTTAATCACCATGCCTCGA-3') and mit133 (5'-GCAAATGGGACATCTCGATGGACT-3'), were used to sequence DNA in both strands. DNA sequence data were analyzed using GENETYX-MAC software for multiple sequence alignment. Phylogenetic analysis was made using the PHYLIP program package, version 3.5c [4]. A dendrogram was made using the neighbor joining (NJ) method [9] from the estimated distance matrices. To determine the confidence intervals of the phylogenies, the bootstrap method [3] was used from 1,000 replications.

The 600-bp mtDNA D-loop region was determined from 365 specimens, and their sequence alignments were shown in Fig. 1. Sixty-four different haplotypes including 18 haplotypes previously detected by Okumura *et al.* [8] were found in the 365 dogs. Forty-six mtDNA haplotypes were newly detected in this study, and their DNA sequences have been deposited in the DDBJ/EMBL/GenBank database (Accession Nos. AB055010 to AB055055). Among 600-bp sequences determined, three nucleotide gaps (nucleotide positions 4, 476, and 477) were found, but no nucleotide insertions were detected in 600-bp sequences from 365 dogs.

Table 1 shows the distributions of mtDNA haplotypes in nine representative breeds detected more than ten specimens. The breeds, Shih-Tzu (79/93; 85%) and Golden Retriever (14/19; 74%) dominantly had the same mtDNA haplotype, III-10. The breeds Siberian Husky and Pomera-

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                 1111111111111111111222222233333333344445555 5
               2357890345566677788889999145567245566788057700477 8
              4742068210670560270348357801051565290186737670480 83
Ⅰ-26*         -CACCCATACTTCTCTACATAAATGATCACCCCTCTTTCCGGCAGCCACAC
Ⅰ-29          -.........................C.....................
Ⅰ-28          -.....G...........................................
Ⅰ-27          -.....G.....................G.................
Ⅰ-20          -.............GT...........................
Ⅰ-18*         -.............G...G.........................
Ⅰ-21          -.............G................C.............
Ⅰ- 6          -...........C..G.............................
Ⅰ-19          -.............G.........................-..T..G.
Ⅰ- 4          -...........C..G.............................T..
Ⅰ-12*         -.............G.......A.....................T..
Ⅰ-13          -..........A...G.......A.....................T..
Ⅰ-17*         -T............G.....................T.....T..
Ⅰ-2           -...........C..G.........................T..T..
Ⅰ-15          -..T.........G.........................T..T..
Ⅰ-11*         -.............G.......A.C...................T..
Ⅰ-46          -...T.........G...G.............C..........T..
Ⅰ-16*         -T............G.............................T..
Ⅰ-14*         -.............G.........................T..T..
Ⅰ-10          -.............G.......A.C...........T........T..
Ⅰ- 3          -...........C..G.........C................T..T..
Ⅰ- 7          -...........C..G.....................T.........
Ⅰ- 1*         -...........C.CG.........................T..T..
Ⅰ- 9          -.............G..C....A.C...........T........T..
Ⅰ-30          -..T...C.....................................T..
Ⅰ-24          -..T.........T..........C.....T.T............T..
Ⅰ-23*         -............T..........C.....T.T............T..
Ⅰ-22*         -...........................T.T............T..
Ⅰ-32          -................T.............................
Ⅰ-39*         -................T.............................T..
Ⅰ-45          -............C....T.........................T..
Ⅰ-36          -.........C.........T.........................T..
Ⅰ-33          -.G..............T.........................T..
Ⅰ-34          -.GT.............T.........................T..
Ⅰ-37*         -...............G.T.........................T..
Ⅰ-38*         -............T.....T.........................T..
Ⅰ-40          -.......T.........T.........................T..
Ⅰ-44          -................T....G...........T.........T..
Ⅰ-43*         -................T.............C...........T..
Ⅰ-41*         -................CT..........................T..
Ⅳ- 4          -..TTA....C.........G.C..........C........T..T.GT..
Ⅳ- 9          -..TT.....C.........G.C..........C........T..T.GT..
Ⅳ- 3          -..TT.....C.........G.C......T...C........T..T.GT..
Ⅳ- 2*         -..TT.....C.........G.C......T...C........T.-T.GT..
Ⅳ- 5          -..TT.....C.........G.CC.........C........T.-T.GT..
Ⅳ- 7          -..TT.....C.........G..C.........C........T.-T.GT..
Ⅳ- 8          -..TT.....C.........G..C....C....C........T.-T.GT..
Ⅳ- 6          -..TT.....C.........G..C...G.....C........T..T.GT..
Ⅲ-21          -...T......C.....T..GG..............C.....T..T.GT..
Ⅲ-19          -...T......C.....T..GG..A........C..C.....T..T.GT..
Ⅲ-17*         C...T......C.....T..GG..A........C..C.....T..T.GT..
Ⅲ-18          C...T......C.....T..GG..A........C..C....TT..T.GT..
Ⅲ-20          -...T......C.....T..GG..A........C..C.....T-.TTGT..
Ⅲ- 4          -...T....T.C.....T..GG..A........C..C.....T-.T.GT..
Ⅲ- 7          -...T....T.C.....T..GG.TA........C..C.....T..T.GT..
Ⅲ-12          -..TT....T.C.....T..GG..A........C..C.....T..T.GT..
Ⅲ- 9          -...T....T.C.....T..GG..A........C..C...T.T..T.GT..
Ⅲ-10*         -...T....T.C.....T..GG..A........C..C.....T..T.GT..
Ⅲ-16          -...T....T.C........GG..A........C..C.....T..T.GT..
Ⅲ- 6          -...T....T.C.....T..GG..A......T.C..C.....T..T.GT..
Ⅲ- 5          -...T....T.C.....T..GG..A........C..C.....T-.T.GT..
Ⅲ-14          -...T....T.C.....T..GG..A........C..C.....T.-T.GT.T
Ⅲ- 8          -...T....T.C.....T..GG..A........C..C...TAT..T.GT..
Ⅲ-15          -...T....T.C.....T..GG..A........C..CA....T..T..T..
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Fig. 1. Base substitutions and gaps found in a survey of the mtDNA D-loop region of 365 dogs. Sixty-four different types are identified and listed. Dots indicate matching with the reference sequence of the Shiba 1, and nucleotide position 4 corresponds to the base position 4 in the complete dog mtDNA D-loop region described by Okumura *et al.* [8]. Asterisks on haplotypes were detected by Okumura *et al.* [8].

Table 1. Dog breeds and their mtDNA haplotypes

Haplotypes[a)]	No. of dog breeds[b)]										
	S.T.	Mal.	Cava.	S. hus.	L.ret.	G.ret	Shet.	Pome.	Pug	Others	Total
I-26*	1	-	-	6	-	-	1	-	-	14	22
III-17*	-	1	-	-	-	-	-	-	-	-	1
I-2	1	1	7	-	3	-	-	-	9	11	32
I-28	-	-	5	-	-	-	-	-	-	-	5
I-41	-	-	3	-	-	-	-	-	-	-	3
I-44	-	-	2	-	-	-	-	-	-	-	2
I-27	-	-	1	-	-	-	-	-	-	-	1
III-4	-	-	1	-	-	-	-	-	-	1	2
III-15	-	-	-	-	-	2	-	-	-	-	2
I-4	-	-	-	-	3	1	-	-	-	3	7
III-8	-	-	-	-	1	-	-	-	-	-	1
I-6	-	-	-	-	1	1	-	-	-	1	3
I-7	-	-	-	-	1	-	-	-	-	-	1
IV-4	-	2	-	-	-	-	-	-	-	-	2
I-34	-	1	-	-	-	-	-	-	-	-	1
IV-9	-	1	-	1	-	-	-	-	-	-	2
I-36	-	1	-	-	-	-	-	-	-	-	1
I-33	-	1	-	-	-	-	-	-	-	-	1
IV-5	-	1	-	-	-	-	-	-	-	-	1
IV-7	-	1	-	-	-	-	-	-	-	3	4
III-19	-	1	-	-	-	-	-		-	1	2
I-43*	-	-	-	-		-	-	5	-	10	15
I-45	-	-	-	-	-	-	-	1	-	1	2
IV-8	-	-	-	-	-	-	-	1	-	-	1
I-14*	-	-	-	-	-	-	4	-	-	4	8
I-40	-	-	-	-	-	-	1	-	-	4	5
I-1*	-	-	-	-	-	-	3	-	-	2	5
III-10*	79	3	-	-	1	14	-	-	-	17	114
I-22*	6	-	-	-	-	-	-	-	-	-	6
IV-3	1	3	-	-	-	1	-	-	-	-	5
I-16	1	-	-	-	-	-	-	-	-	-	1
I-39*	2	13	1	-	2	-	-	6	2	5	31
III-6	1	-	-	-	-	-	-	-	-	-	1
III-5	1	-	-	-	-	-	-	-	-	2	3
I-12*	-	-	-	7	-	-	-	-	-	2	9
I-13	-	-	-	2	-	-	-	-	-	-	2
I-17*	-	-	-	-	-	-	1	-	-	2	3
Others	-	-	-	-	-	-	-	-	-	58	58
Total	93	30	20	16	12	19	10	13	11	141	365

a) Haplotypes with asterisks are found in Okumura *et al.* [8].
b) Abbreviations: S.T., Shih Tzu, Mal., Maltese; Cava., Cavalier King Charles Spaniel; S. hus., Siberian Huskey; L. ret., Labrador Retriever; G. ret., Golden Retriever; Shet., Shetland sheepdog; Pome., Pomeranian. Other, forty dog breeds and mongrel.

nian had mtDNA haplotypes I-26 and I-12, and I-43 and I-39, respectively (Table 1). The Maltese had 13 kinds of mtDNA haplotypes, with haplotype I-39 the most dominant. Seven mtDNA haplotypes were found in the Cavalier King Charles Spaniel, with I-2, I-28, and I-41 mtDNA haplotypes predominant among them (Table 1).

Nucleotide variations in 64 haplotypes detected in this study and 15 haplotypes previously reported [8] was calculated using a two-parameter method [7]. A dendrogram was constructed using the NJ method (Fig. 2). The diversity of mtDNA 600-bp haplotypes was classified into four clusters I to IV, as previously reported [8]. The mtDNA haplotype III-10 predominantly found in the breeds Shih-Tzu and Golden Retriever was classified into cluster III. Thirteen mtDNA haplotypes detected in the Maltese comprised five haplotypes in cluster I, three haplotypes in cluster III and five haplotypes in cluster IV, suggesting that the Maltese was a breed established from different gene pools. As most dog breeds were found in the cluster I, dogs belonging to the cluster I may contribute to the variation of several dog breeds as a gene pool. However, no dog belonging to cluster II was found in this study, suggesting that mtDNA haplotypes II-1 and II-2 in the cluster II are seldom haplotypes in the dog breeds of today.

In this study, the examined dogs from 4 to 9 years (158/362: 43%) were most dominant among 365 patient dogs,

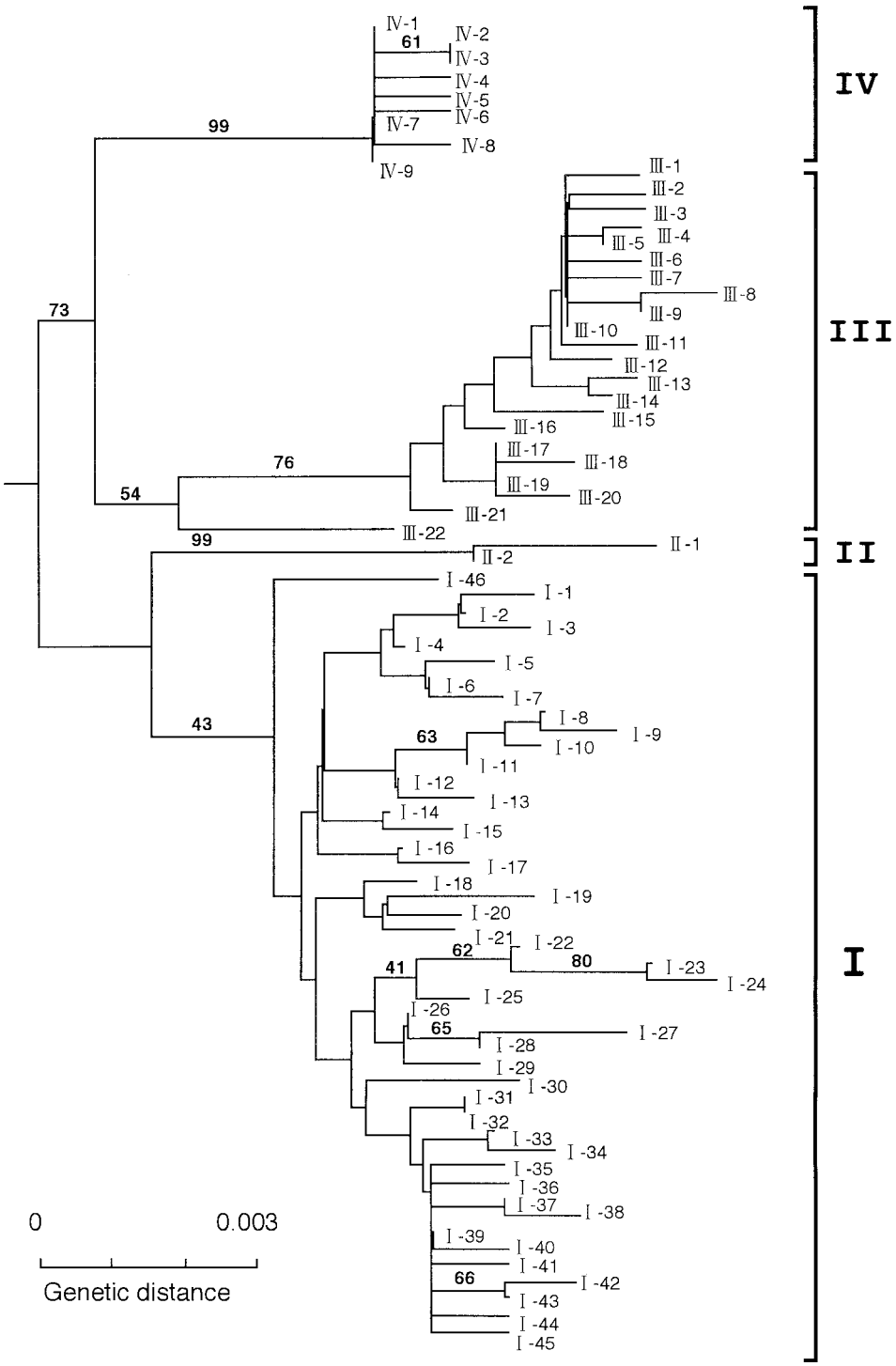

Fig. 2. Neighbor joining tree drawn from indices of the nucleotide substitution at each site among 365 dog mtDNA sequences calculated using the Kimura 2-parameter method [7]. Designation of each haplotype is as shown in Fig. 1; the other 15 haplotypes detected by Okumura *et al.* [8] are included. The nucleotide sequences of the 15 haplotypes from the Okumura's paper [8] are as follows: IV-I, the Shikoku124; III-1, the Shih Tzu40; III-2, the Saluki15; III-3, the Hokkaido108; III-11, the Ryukyu131; III-13, the Ryukyu126; III-22, the Shiba78; II-1, the Siberian Husky102; II-2, the Kishu25; I-5, the Kishu38; I-8, the Ryukyu129; I-25, the Afgan120; I-31, the Pointer174; I-35, the Eskimo183; I-42, the Hokkaido123. This tree is based on 600-bp of the mtDNA D-loop region. Numbers on the branch indicate bootstrap values (%, 1,000 replicates). Dog haplotypes are grouped into four clusters I to IV.

and puppies under 1 year and dogs older than 14 years were few. The number of male and female dogs examined were almost the same. The genetic relationship between mtDNA haplotypes in each dog breed and canine disorders was examined in the dogs with clinical diagnosis. Among the 91 dogs with dermatitis, haplotypes III-10, I-1 and III-10 were predominant in the Shih-Tzu (35 dogs), the Cavalier King Charles Spaniel (3 dogs) and the Golden Retriever (6 dogs), respectively. Mitral regurgitation was found in mtDNA haplotype III-10 (6 dogs) in the Shih Tzu and I-39(5 dogs) in the Maltese, and canine hip dysplasia was specific for the Labrador Retriever with haplotypes III-10, I-39 and I-2. Mammary gland tumor was found in dog breeds with different mtDNA haplotypes. However, no disorder closely associated with mtDNA haplotypes was found in any dog breeds.

Schafer *et al.* [10] reported that a family susceptible to mammary neoplasia had a markedly younger age of onset and had different ratios of benign to malignant tumors, indicating that a certain lineage within a dog breed shows a familial predisposition to mammary neoplasia. This result indicates that lineage analyses using the mtDNA haplotype or microsatellite analyses will be helpful to select target animals or to acquire experimental model animals. Therefore, more detailed clinical informations and nuclear mutation markers more directly associated with the diseases should be collected to identify dog lineage influencing the familial disorders.

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