Mitochondrial DNA Polymorphism in Dogs

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ABSTRACT. Mitochondrial DNA (mtDNA) polymorphism was studied in 20 mongrel dogs using 14 restriction enzymes. The polymorphism was observed in the cleavage patterns of *Apa* I, *Eco*R I, *Eco*R V, *Hinc* II and *Sty* I. Three morphs using *Eco*R I and *Apa* I and two morphs using *Eco*R V, *Hinc* II and *Sty* I were found. However, no polymorphism was detected in the cleavage patterns of *Bam*H I, *Bgl* II, *Hae* II, *Hind* III, *Pst* I, *Sal* I, *Sca* I, *Stu* I and *Xba* I. The examined dogs were classified in seven types using five restriction endonucleases which recognized six nucleotide sequences. The value of nucleotide diversity was estimated to be 0.0055. A phylogenetic tree constructed by genetic distances among seven restriction types showed at least two clusters of mtDNA.—KEY WORDS: dog, mitochondrial DNA (mtDNA), polymorphism, restriction endonuclease.

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Mammalian mitochondrial DNA (mtDNA) is a closed circular molecule that contains approximately 16.5 k base pairs(kb). mtDNA is maternally inherited, which has been demonstrated in human [15] and other mammalians [21, 32]. The complete nucleotide sequences of mammalian mtDNA genome have been demonstrated in human [1], cow [2], mouse [4] and rat [14]. The genes for the 12S and 16S rRNAs, 22 tRNAs, cytochrome c oxidase subunits I, II and III, ATPase subunit 6, cytochrome b and eight unidentified proteins have located [1, 2, 4]. The arrangement of mitochondrial genes is highly conserved among mammalian animals [1, 2, 4, 14]. However, despite this conservative feature, nucleotide sequences of mtDNA have shown to evolve rapidly [7, 8]. The rate of evolution of mitochondrial genome appears to be 5–10 times higher than that of single-copy nuclear DNA [7, 8]. Because of these evoluting properties, its reduced size, high copy number and unsexual mode of inheritance, mtDNA has become a very powerful tool for studies of evolutional genetics.

Since substantial sequence variation among individuals was reported in mtDNA, mtDNA polymorphism has been analyzed widely in human and several animal species not only using restriction endonucleases but also by determining nucleotide sequences [6, 7, 10, 13, 18–20, 25–30, 33]. The results obtained from analyses using several restriction endonucleases show a high correlation between mtDNA types and human ethnic origin [5, 11, 12, 16, 22]. In addition to point mutations which indicate a change of restriction site in mtDNA, length mutation also occurs [9, 17].

Several animal species have been analyzed for mtDNA polymorphisms using restriction endonucleases [3, 25, 26, 28–31, 33]. However, there are few data concerning mtDNA in dogs. In the present paper, we studied mtDNA polymorphism in dogs with 14 restriction endonucleases that recognized six base pairs.

MATERIALS AND METHODS

mtDNA isolation: mtDNA was prepared from the kidneys of dogs by the method of Lansman *et al.* [23] with slight modifications. The kidneys which were obtained from mongrel dogs by autopsy were minced on a chilled glass plate on ice and homogenized in buffer containing 0.21 M mannitol, 0.07 M sucrose, 0.05 M Tris-HCl pH 7.5 and 3 mM $CaCl_2$. After homogenization, disodium EDTA at pH 7.5 was added to a final concentration of 10 mM. Nuclear and cell debris were removed from the homogenate by centrifugation at 700 × g for 5 min. The centrifugation was repeated. Mitochondria was then pelleted by centrifugation at 20,000 × g for 20 min. The pellets were washed by resuspending it in the buffer solution used for homogenization containing 0.01 mM EDTA instead of 3 mM $CaCl_2$ and recentrifugation at 20,000 × g for 20 min. Mitochondrial pellets were resuspended in 3 m*l* of the buffer solution containing 0.1 M NaCl, 0.05 M Tris-HCl pH 8.0 and 0.01 M EDTA and lysed by the addition of 0.15 m*l* of 25% sodium dodecyl sulfate (SDS) and incubation for 5 min at 37°C. The lysates

were cooled on ice for 1 hr after addition of 0.5 m*l* of 7 M CsCl solution to precipitate high molecular weight DNA and protein. The precipitate was then removed by centrifugation at 20,000 × g for 10 min. After the addition of ethidium bromide to 500 mg/m*l*, the density of the lysates were adjusted to 1.58 g/cm^3 by adding CsCl. The lysate was then centrifuged for 18 hr at 20°C. After centrifugation, the lower band of mtDNA was collected by puncturing the side of the tube under UV illumination. mtDNA was rebanded by CsCl-ethidium bromide density gradient ultracentrifugation. The mtDNA samples were extracted three times with butanol to remove ethidium bromide and the DNA was precipitated with 2.2 volume of 95% ethanol.

Restriction endonuclease digestion analysis: The purified mtDNA from each samples was digested with 14 restriction endonucleases recognizing six base pairs. The restriction endonucleases used were *Apa* I, *Bam*H I, *Bgl* II, *Eco*R I, *Eco*R V, *Hae* II, *Hind* III, *Hinc* II, *Pst* I, *Sal* I, *Sca* I, *Stu* I, *Sty* I and *Xba* I. Enzymes were purchased from Takara Shuzo Co., Japan and Nippon Gene Co., Japan. Digestions were performed according to the supplier's directions. Restriction fragments were separated on horizontal agarose gel and visualized under UV light by ethidium bromide staining. Alternatively, the products of digestion were transferred to nylon membrane and hybridized with ^{32}P labeled dog mtDNA as a probe. After hybridization, the membrane was thoroughly washed and subjected to autoradiography.

Nucleotide diversity: The average amount of nucleotide change in dog mtDNA was estimated by the method of cleavage site comparison. First, the population ($\hat{S}$) of shared restriction site between any two restriction types was determined by using equation (10) of Nei and Li [24]: $\hat{S} = 2\,Nxy/(Nx+Ny)$, where Nx and Ny are the number of restriction sites in type x and y, respectively, and Nxy is the number of restriction site shared by types x and y. The number (d) of nucleotide substitutions per nucleotide site was then estimated by a equation (9) of Nei and Li [24]: $d = -(3/2)\ln[(4\hat{S}^{1/2r}-1)/3]$, where r is the number of nucleotides of the recognition sequence for restriction enzymes used. In this study, r is equal to 6.

RESULTS

Cleavage patterns with restriction eyzmes: Figure 1 shows typical dog mtDNA cleavage patterns digested by using 14 restriction endonucleases analyzed by agarose gel electrophoresis.

*Eco*R I: When individual mtDNA from 20 dogs was digested with restriction endonuclease *Eco*R I, three different cleavage patterns were observed (Fig. 2). Morph 1 which was the dominant pattern showed three fragments of approximately 8.0, 7.5 and 0.6 kb. Morph 2 and morph 3 were derived from morph 1. Morph 2 showed four fragments and differed from morph 1 by the loss of a 7.5 kb fragment to yield two smaller fragments of 5.15 and 2.35 kb. Morph 3 showed four fragments and

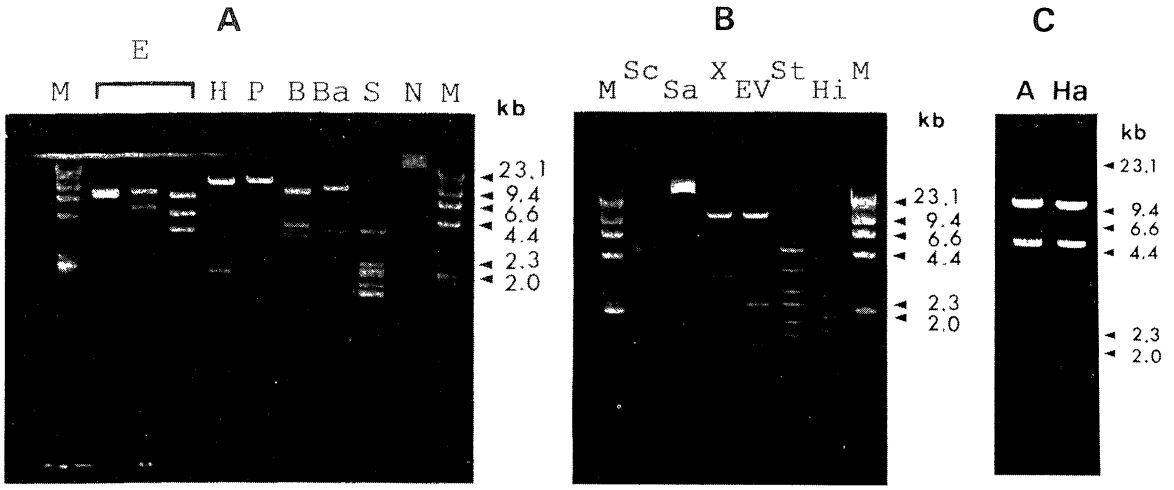

Fig. 1. Comparison of 14 restriction enzyme cleavage patterns of mtDNA in dogs analyzed by 1.4% agarose gel electrophoresis. Fragments were visualized by ethidium bromide staining and UV light. (A) E: *Eco*R I, H: *Hind* III, P: *Pst* I, B: *Bgl* II, Ba: *Bam*H I, S: *Sty* I, N: non-digested mtDNA, M: marker DNA (B) Sc: *Sca* I, Sa: *Sal* I, X: *Xba* I, EV: *Eco*R V, St: *Stu* I, Hi: *Hinc* II, M: marker DNA (C) A: *Apa* I, Ha: *Hae* II.

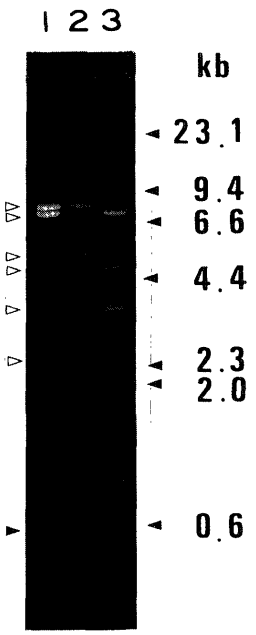

Fig. 2. *Eco*R I cleavage patterns of dog mtDNA separated on 0.8% agarose gel and visualized under UV light by ethidium bromide staining. Morphs are donated by numbers at top of lanes and fragment sizes are designated in kilobases. Open triangles show polymorphic fragments and a solid triangle shows common fragment. The undigested mtDNA is also visualized on lane 1.

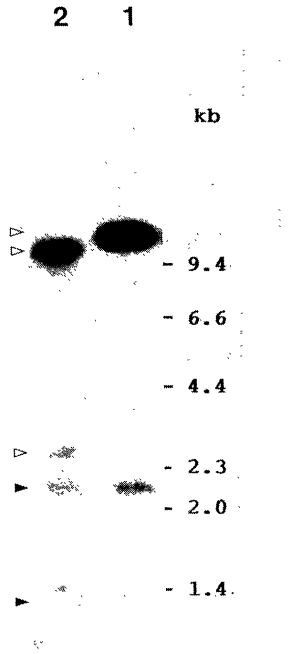

Fig. 3. *Eco*R V cleavage patterns of dog mtDNA separated on 1.4% agarose gel. The fragments were visualized by southern hybridization method using ^{32}P labeled purified dog mtDNA as a probe and autoradiography. Morphs are donated by numbers at top of lanes and fragment sizes are designated in kilobases. Open triangles show polymorphic fragments and solid triangles show common fragments.

differed from morph 1 by the loss of a 8.0 kb fragment to yield two smaller fragments of 4.6 and 3.4 kb. Morph 2 and morph 3 gained an *Eco*R I site in 7.5 and 8.0 kb fragments in morph 1, respectively.

*Eco*R V: Two different morphs were observed in our samples by the digestion with *Eco*R V (Fig. 3). Morph 1 was the dominant pattern which was consist of three fragments of approximately 12, 2.15 and 1.38 kb showing faint density. Morph 2 was generated by the loss of the largest fragment of 12 kb in morph 1 and the appearance of two smaller fragments of 9.5 and 2.5 kb. Morph 2 gained an *Eco*R V site in the 12 kb fragment in morph 1.

Hinc II: Two different morphs were found (Fig. 4). Morph 1 was the dominant pattern and showed six fragments of approximately 3.3, 2.4, 1.95, 1.85, 1.6 and 1.5 kb. Morph 2 differed from morph 1 by the fusion of the 3.3 kb fragment to the 1.6 kb fragment to yield a new fragment of 4.9 kb. Morph 2 was shown to be derived from morph 1 by the loss of a single *Hinc* II site.

Sty I: Two different morphs were observed (Fig. 5). Morph 1 showed seven fragments of approximately 3.8, 2.25, 2.0, 1.9, 1.65, 1.5 and 0.76 kb. Morph 2 also showed seven fragments. Six fragments except the 2.0 kb fragment were detected in two morphs. In morph 2, an approximately 2.1 kb fragment was detected instead of the 2.0 kb fragment observed in morph 1. Morph 2 seemed to be generated by a site loss in the 2.1 kb fragment.

Apa I: Three different morphs were observed (Fig. 6). Morph 1 was the dominant pattern which showed two fragments estimated to be 11.6 and 4.7 kb. Morph 2 was generated by the loss of the largest fragment in morph 1 and the appearance of three smaller fragments of approximately 6.9, 3.5 and 1.2 kb. Morph 2 was found to have gained two *Apa* I sites in the 11.6 kb fragment in morph 1. On the other hand, morph 3 was a confusing pattern which showed only two fragments of four of those in morph 2. For analysis of smaller fragments produced by the digestion of mtDNA with *Apa* I,

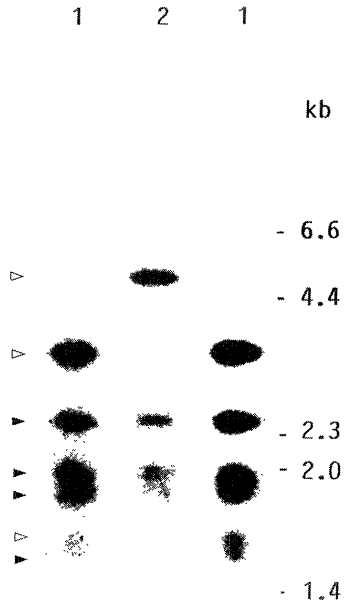

Fig. 4. *Hinc* II cleavage patterns of dog mtDNA separated on 1.8% agarose gel. The fragments were visualized by southern hybridization method using ^{32}P labeled purified dog mtDNA as a probe and autoradiography. Morphs are donated by numbers at top of lanes and fragment sizes are designated in kilobases. Open triangles show polymorphic fragments and solid triangles show common fragments.

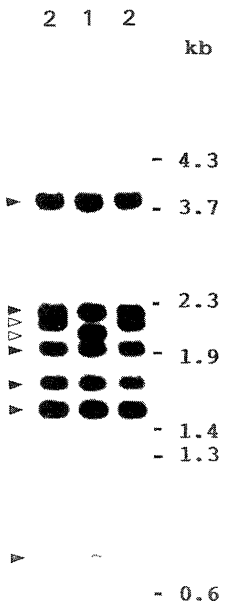

Fig. 5. *Sty* I cleavage patterns of dog mtDNA separated on 1.4% agarose gel. The fragments were visualized by southern hybridization method using purified mtDNA as a probe. Morphs are donated by numbers at top of lanes and fragment sizes are designated in kilobases. Open triangles show the polymorphic fragments and solid triangles show common fragments.

polyacrylamide gel electrophoresis and silver staining were carried out. The analysis of small size mtDNA fragments showed that no fragments which were smaller than the 1.2 kb fragment observed in morph 2 were detected by polyacrylamide gel electrophoresis and silver staining (not shown in figure). There was no evidence of deletions during the course of this analysis. The simplest interpretation of the *Apa* I digestion pattern of morph 3 is that a site gain occurs in the 11.6 kb fragment of morph 1 and two smaller fragments are produced. These fragments are estimated to be 6.9 and 4.7 kb. However, the approximately 4.7 kb fragment overlaps 4.7 kb fragment in morph 1. The overlapped fragments are not separated in our condition. Thus, morph 3 shows three fragments which are 6.9, 4.7 and another 4.7 kb fragments. To confirm this interpretation, double digestion tests were carried out (Fig. 7). The samples which showed morph 3 were digested with *Apa* I and another enzymes, *Pst* I, *Hind* III or *Xba* I, which showed no polymorphisms. There were one, two and two restriction sites of *Pst* I, *Hind* III and *Xba* I, respectively. When mtDNA was digested with *Apa* I and *Pst* I, three fragments estimated to be approximately 6.9, 4.7 and 4.6 kb were observed. *Apa* I-*Hind* III digestion showed five fragments estimated to be 6.9, 4.7, 2.1, 1.8 and 0.8 kb. *Apa* I-*Xba* I digestion showed four fragments of 6.9, 4.7, 3.1 and 1.6 kb. These double digestion tests ensured that morph 3 of *Apa* I consisted of three fragments. Two of the three were estimated to be 4.7 kb and overlapped. Morph 3 was derived from morph 1 by the gain of an *Apa* I site and morph 2 derived from morph 3 by the gain of an *Apa* I site. Nine restriction enzymes, *Bam*H I, *Bgl* II, *Hae* II, *Hind* III, *Pst* I, *Sal* I, *Sca* I, *Stu* I and *Xba* I, out of 14 enzymes used in this study showed no polymorphism. Dog mtDNA has three, three, two, two, one, three, six and two sites of *Bam*H I, *Bgl* II, *Hae* II, *Hind* III, *Pst* I, *Sca* I, *Stu* I and *Xba* I, respectively. However, no *Sal* I site was found in dog mtDNA.

Distribution: The frequencies of all morphs observed in our samples are summarized in Table 1.

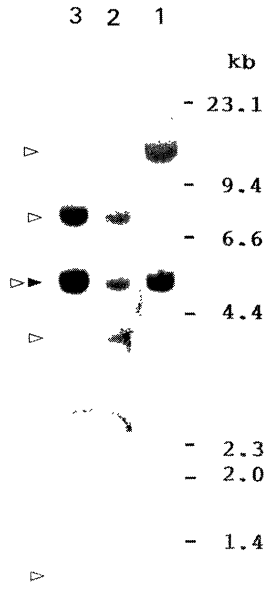

Fig. 6. *Apa* I cleavage patterns of dog mtDNA separated on 0.9% agarose gel. The fragments were visualized by southern hybridization method using purified mtDNA as a probe. Morphs are donated by numbers at top of lanes and fragment sizes are designated in kilobases. Open triangles show the polymorphic fragments and solid triangles show common fragments.

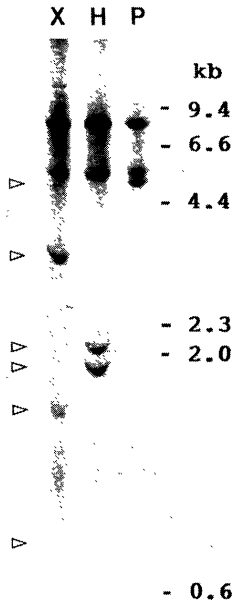

Fig. 7. Double digestion test for analyzing the overlapped fragments of morph 3 in *Apa* I digestion. P, H and X at the top of lane mean the double digestion with *Apa* I and *Pst* I, *Hind* III and *Xba* I, respectively. Original *Apa* I-morph 3 have 6.9 kb and 4.7 kb fragments. Open triangles show the generated fragments after double digestion. Double digestion with *Apa* I and Pst I newly generated 4.6 kb fragment. *Apa* I-*Hind* III and *Apa* I-*Xba* I digestion showed three fragments (2.1 kb, 1.8 kb and 0.8 kb) and two (3.1 kb and 1.6 kb) fragments besides original *Apa* I-morph 3 fragments. The newly generated fragments were found to be derived from a band of the overlapped 4.7 kb fragments in *Apa* I-morph 3.

Table 1. Number and frequencies of mtDNA morphs in dogs

Enzymes	Morphs	No. of dogs	Frequencies (%)
Apa I	1	9	45.0
	2	7	35.0
	3	4	20.0
*Eco*R I	1	16	80.0
	2	2	10.0
	3	2	10.0
*Eco*R V	1	16	80.0
	2	4	20.0
Hinc II	1	17	85.0
	2	3	15.0
Sty I	1	16	80.0
	2	4	20.0
*Bam*H I	1	20	100.0
Bgl II	1	20	100.0
Hae III	1	20	100.0
Hind III	1	20	100.0
Pst I	1	20	100.0
Sal I	1	20	100.0
Sca I	1	20	100.0
Stu I	1	20	100.0
Xba I	1	20	100.0

All five restriction enzymes which showed polymorphism were found to be useful to detect polymorphism of mtDNA, because 15% of individuals showed atypical cleavage patterns at least. *Apa* I was found to be the most useful because the frequency of the digested patterns with *Apa* I was not biased in one typical morph. By combining the restriction endonuclease morphs observed for each of the 20 individuals, we found seven distinct mtDNA types which are listed by frequency in Table 2. The dominant type with the highest frequency was type 5. However, only 35% of individuals showed this dominant type. The frequency of the dominant type in this study was lower than that of human and other animal species by combining the six base pair recognizing restriction enzyme morphs.

Table 2. mtDNA restriction types based on all morphs observed in 20 dogs using 14 restriction enzymes

Types	Enzyme morphs														Observed	
	Apa I	*Eco*R I	*Eco*R V	*Hinc* II	*Sty* I	*Bam*H I	*Bgl* II	*Hae* II	*Hind* III	*Pst* I	*Sal* I	*Sca* I	*Stu* I	*Xba* I	No.	%
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	15 0
2	1	1	2	2	2	1	1	1	1	1	1	1	1	1	3	15.0
3	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2	10.0
4	1	3	2	1	2	1	1	1	1	1	1	1	1	1	1	5.0
5	2	1	1	1	1	1	1	1	1	1	1	1	1	1	7	35.0
6	3	1	1	1	1	1	1	1	1	1	1	1	1	1	3	15.0
7	3	3	1	1	1	1	1	1	1	1	1	1	1	1	1	5.0
Total															20	100.0

Nucleotide diversity: The results obtained with the various restriction endonucleases were also used to compute nucleotide diversity. The average amount of nucleotide change in dog mtDNA could be estimated by the method of cleavage site comparison described by Nei and Li [24]. In this study, an average of 48 sites equivalent to 288 base pairs was compared. The mean value was estimated to be 0.0055.

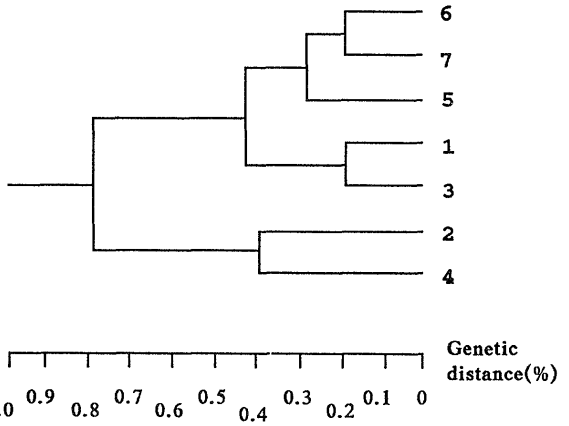

Fig. 8. A phylogenetic tree depicted by the Unweighted Pair-Group method based on genetic distances for seven types in dogs.

DISCUSSION

mtDNA polymorphism in dogs was observed using five restrictiion endonucleases, *Apa* I, *Eco*R I, *Eco*R V, *Hinc* II and *Sty* I. In the cleavage patterns of mtDNA in dogs, *Apa* I showed an embarrassing pattern. Morph 3 consisted of only two bands, which were two of the four bands found in morph 2. No evidence of deletion or heterogeneity was obtained. Double-digestion analyses indicated that morph 3 consisted of three fragments, which were estimated to be 6.9, 4.7 and another 4.7 kb fragments.

These morphs using *Eco*R I and *Apa* I and two morphs using *Eco*R V, *Hinc* II and *Sty* I were found in the present study. All morphs could be explained as being derived one from the other by single changes believed to be point mutation. In the *Eco*R I cleavage patterns, the original morph is found to be morph 1. Morph 2 and morph 3 seem to be derived from morph 1 by the gain of a single *Eco*R I site, respectively. In *Apa* I cleavage patterns, morph 2 is found to be the original pattern because of the highest frequency in mtNDA restriction types based on all morphs observed in 20 dogs using 14 restriction enzymes. Morph 3 is derived from morph 2 by the loss of a single *Apa* I site and morph 1 is from morph 3 by the loss of a single *Apa* I site. In the cleavage patterns with three other restriction endonucleases, *Eco*R V, *Hinc* II and *Sty* I, the dominant patterns which are designated morph 1 are found to be the original patterns.

mtDNA has been demonstrated to be an excellent tool for the study of evolutionary genetics in animals and human [6, 7, 12, 13, 16, 22, 27, 30]. The present study shows that mtDNA is also a useful tool for genetic studies in dogs. The 20 examined dogs were classified into seven types using five restriction endonucleases which recognized six nucleotide sequences. The value of nucleotide diversity in dogs was calculated to be 0.0055. The reported values for human population were 0.0036 by Brown [6] and 0.00417 by Horai *et al.* [18]. The value of the nucleotide diversity in dogs was higher than those of human.

The information from combining the restriction endonuclease morphs observed for each of the 20 dogs in Table 2 was used to construct a phylogenetic tree of dog mtDNA types. A phylogenetic tree constructed by genetic distances among the seven

restriction types in the 20 dogs is shown in Fig. 8. In the tree, at least two clusters of mtDNA types were observed. One of the clusters consisted of two types, which were type 2 and type 4. Four of the 20 dogs are apparently far from other dogs in the phylogenetic tree. The relationship between the clusters in a phylogenetic tree and the breed origins were unclear. There was no knowledge about the genetic origin of the mongrel dogs. In human, analyses of mtDNA polymorphism indicated that there was high correlation between mtDNA restriction types and ethnic origins of individuals [5, 10–12, 16, 22]. The relationships among several breeds of livestock have also been shown based on mtDNA restriction endonuclease cleavage patterns [28–30]. We guess that there is relationship between mtDNA polymorphism and the origin of several breeds in dogs. mtDNA polymorphism may become a useful tool for not only studying evolutional genetics but also maintaining the purity of breeds of dogs.

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